Selective cytotoxicity of intracellular amyloid β peptide$_{1-42}$ 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 Aβ$_{1-42}$ 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 Aβ$_{1-42}$ peptide or a cDNA-expressing cytosolic Aβ$_{1-42}$ rapidly induces cell death of primary human neurons. In contrast, Aβ$_{1-40}$, Aβ$_{40-1}$, or Aβ$_{42-1}$ peptides, and cDNAs expressing cytosolic Aβ$_{1-40}$ or secreted Aβ$_{1-42}$ and Aβ$_{1-40}$, are not toxic. As little as a 1-pM concentration or 1500 molecules/cell of Aβ$_{1-42}$ peptides is neurotoxic. The nonfibrillized and fibrillized Aβ$_{1-42}$ peptides are equally toxic. In contrast, Aβ$_{1-42}$ peptides are not toxic to human primary astrocytes, neuronal, and nonneuronal cell lines. Inhibition of de novo protein synthesis protects against Aβ$_{1-42}$ toxicity, indicating that programmed cell death is involved. Bcl-2, Bax-neutralizing antibodies, cDNA expression of a p53^{R273H} dominant negative mutant, and caspase inhibitors prevent Aβ$_{1-42}$-mediated human neuronal cell death. Taken together, our data directly demonstrate that intracellular Aβ$_{1-42}$ 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– (Aβ$_{1-40}$) or 42– (Aβ$_{1-42}$) 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 NH$_2$-terminally truncated form of Aβ$_{1-42}$ (Aβ$_{42}$) accumulates in the ER in aging cell cultures (Greenfield et al., 1999; Yang et al., 1999). Recently, the presence of intracellular Aβ$_{42}$ 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 Aβ$_{42}$ 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 Aβ$_{1-28}$ and Aβ$_{40}$ precedes Aβ$_{42}$, and these intracellular Aβ accumulations precede the appearance of diffuse plaques, senile core plaques, and NFT formation (Gyure et al., 2001). In aging
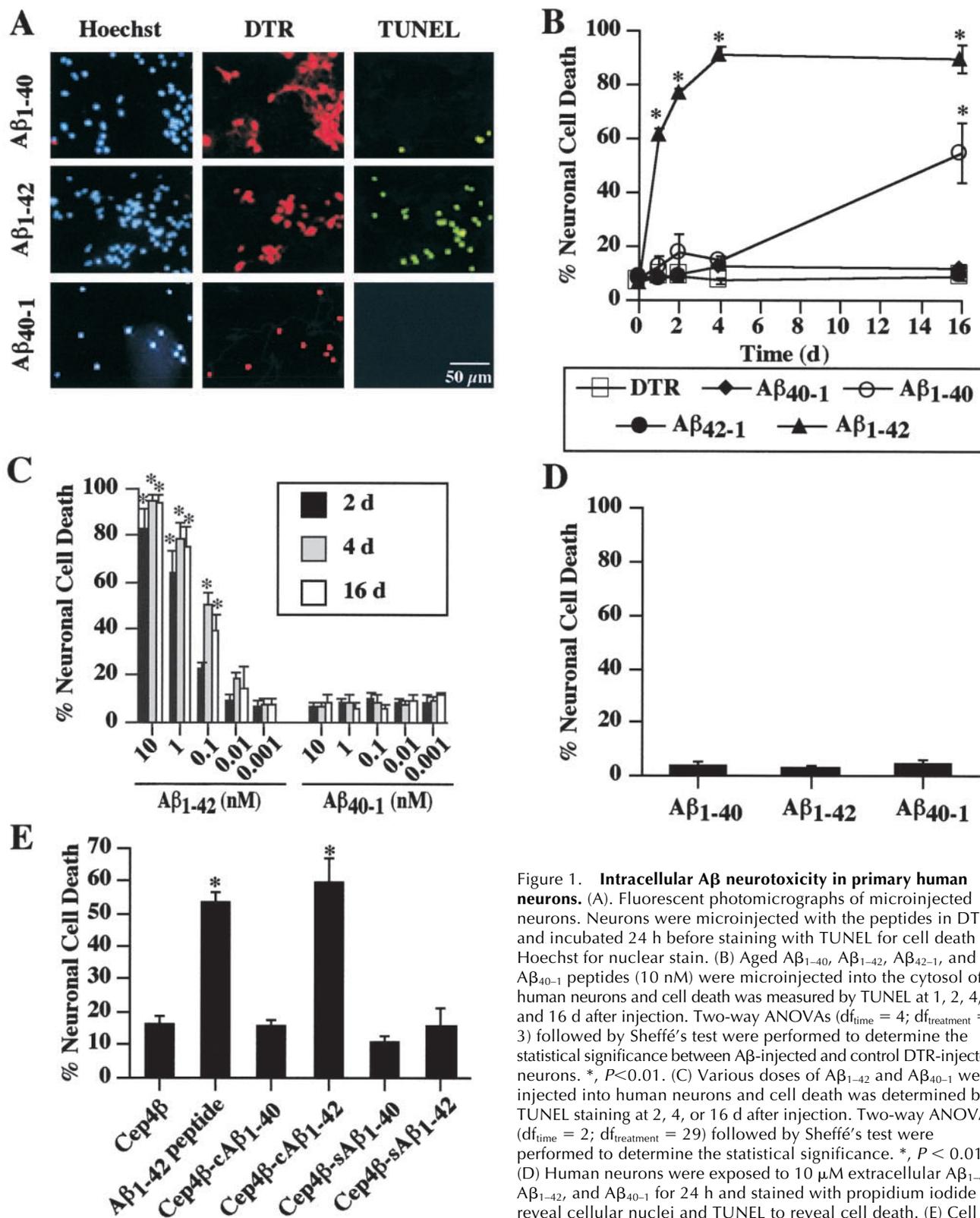
Figure 1. **Intracellular Aβ neurotoxicity in primary human neurons.** (A). Fluorescent photomicrographs of microinjected neurons. Neurons were microinjected with the peptides in DTR and incubated 24 h before staining with TUNEL for cell death or Hoechst for nuclear stain. (B) Aged $A\beta_{1-40}$, $A\beta_{1-42}$, $A\beta_{42-1}$, and $A\beta_{40-1}$ 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 ($df_{time} = 4$; $df_{treatment} = 3$) 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 $A\beta_{1-42}$ and $A\beta_{40-1}$ were injected into human neurons and cell death was determined by TUNEL staining at 2, 4, or 16 d after injection. Two-way ANOVAs ($df_{time} = 2$; $df_{treatment} = 29$) followed by Sheffé's test were performed to determine the statistical significance. *, $P < 0.01$. (D) Human neurons were exposed to 10 μM extracellular $A\beta_{1-40}$, $A\beta_{1-42}$, and $A\beta_{40-1}$ 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 pCep4β episomal cDNA constructs expressing cytosolic $A\beta_{1-40}$ and $A\beta_{1-42}$ (cAβ) or secreted $A\beta_{1-40}$ and $A\beta_{1-42}$ (sAβ). One-way ANOVA ($df = 5$) followed by Sheffé's test determined a statistically significant difference between the Cep4β construct alone and $A\beta_{1-42}$ peptide or Cep4β-c$A\beta_{1-42}$ 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 APP^{V717F} 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 $A\beta_{42}$ 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 $A\beta_{1-40}$, $A\beta_{1-42}$, and control reverse peptides $A\beta_{40-1}$ and $A\beta_{42-1}$ or cDNAs expressing cytosolic or secreted $A\beta_{1-40}$ and $A\beta_{1-42}$ in primary cultures of human neurons, neuronal, and nonneuronal cell lines. $A\beta_{1-42}$ was selectively toxic to human neurons. Microinjections of the $A\beta_{1-42}$ peptide in the presence of transcriptional and translational inhibitors showed that the cytotoxicity of $A\beta_{1-42}$ 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 $A\beta_{1-42}$-mediated neurotoxicity. Both eliminated toxicity. Furthermore, the $p53^{R273H}$ dominant negative mutant, which prevents transactivation of Bax expression (Aurelio et al., 2000), eliminated $A\beta_{1-42}$ toxicity. Lastly, caspase-6–, but not caspase-3–type inhibitors, prevented $A\beta_{1-42}$ toxicity. We conclude that $A\beta_{1-42}$ is selectively toxic to human neurons through activation of the p53 and Bax proapoptotic pathway.

Results

Intracellular $A\beta_{1-42}$, but not $A\beta_{1-40}$, $A\beta_{42-1}$, or $A\beta_{40-1}$, is neurotoxic to human neurons

To directly determine whether intracellular Aβ is toxic to human neurons, we microinjected aged $A\beta_{1-40}$, $A\beta_{1-42}$, or the reverse control peptide $A\beta_{40-1}$ into the cytoplasm of primary cultured human neurons. $A\beta_{1-42}$ 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, $A\beta_{1-40}$, the control reverse peptides, $A\beta_{40-1}$, $A\beta_{42-1}$, 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 $A\beta_{1-40}$ 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 $A\beta_{1-42}$ 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 $A\beta_{1-42}$ 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 $A\beta_{1-42}$, $A\beta_{1-40}$, or $A\beta_{40-1}$, 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 $A\beta_{1-40}$, $A\beta_{1-42}$, or $A\beta_{40-1}$ are toxic to these neurons after 24 h of treatment (Fig. 1 D). Therefore, the toxicity of intracellular $A\beta_{1-42}$ is at least 100,000 times greater than extracellular Aβ. These results indicate that an infinitesimal amount of intracellular $A\beta_{1-42}$ is detrimental to human neurons. Calculation of the number of molecules of $A\beta_{1-42}$ injected in neurons based on the Avogadro number shows maximal toxicity with 150,055 molecules and 50% toxicity with 1505.5 molecules/neuron. The level of toxic $A\beta_{1-42}$ is probably at least 10,000-fold lower than the amount of immunologically detectable intracellular $A\beta_{1-42}$ 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 $A\beta_{1-42}$ 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 $A\beta_{1-40}$ and $A\beta_{1-42}$. As observed with the synthetic $A\beta_{1-42}$ peptide, only the cytosolically expressed $A\beta_{1-42}$ was toxic, whereas secreted $A\beta_{1-42}$ or cytosolic or secreted $A\beta_{1-40}$ did not induce cell death in neurons (Fig. 1 E).

Nonfibrillized $A\beta_{1-42}$ 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 $A\beta_{1-42}$ peptides show well defined globular as well as diffuse aggregate morphology. Similar well-defined globular structures are also seen in the nonfibrillized $A\beta_{1-40}$ preparation, but are much less abundant than in the $A\beta_{1-42}$ preparation. The fibrillized $A\beta_{1-42}$ peptide shows a heterogeneous mixture of fibrils of various sizes, protofibrils, and globular structures. The fibrils in the $A\beta_{1-40}$ preparation were less heterogeneous and consisted of thick fibrillar aggregates and thin aligned fibrils. The large $A\beta_{1-40}$ 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 $A\beta_{1-42}$ and $A\beta_{1-40}$ preparation did pass through the pipette. Both fibrillized and nonfibrillized preparations of $A\beta_{1-42}$ induce 50–90% neuronal cell death between 24–96 h after injection (Fig. 2 B). In contrast, neither fibrillized nor nonfibrillized $A\beta_{1-40}$ 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 $A\beta_{1-42}$ peptide forms aggregates with the expected size of Aβ dimers, trimers, and oligomers. In contrast, the nonfibrillized and fibrillized $A\beta_{1-40}$ 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 $A\beta_{1-42}$ is significantly more toxic than the monomeric $A\beta_{1-40}$ or the dimers, trimers, and oligomers of the nonfibrillized and fibrillized $A\beta_{1-42}$ peptide may be the toxic forms of the Aβ peptides.
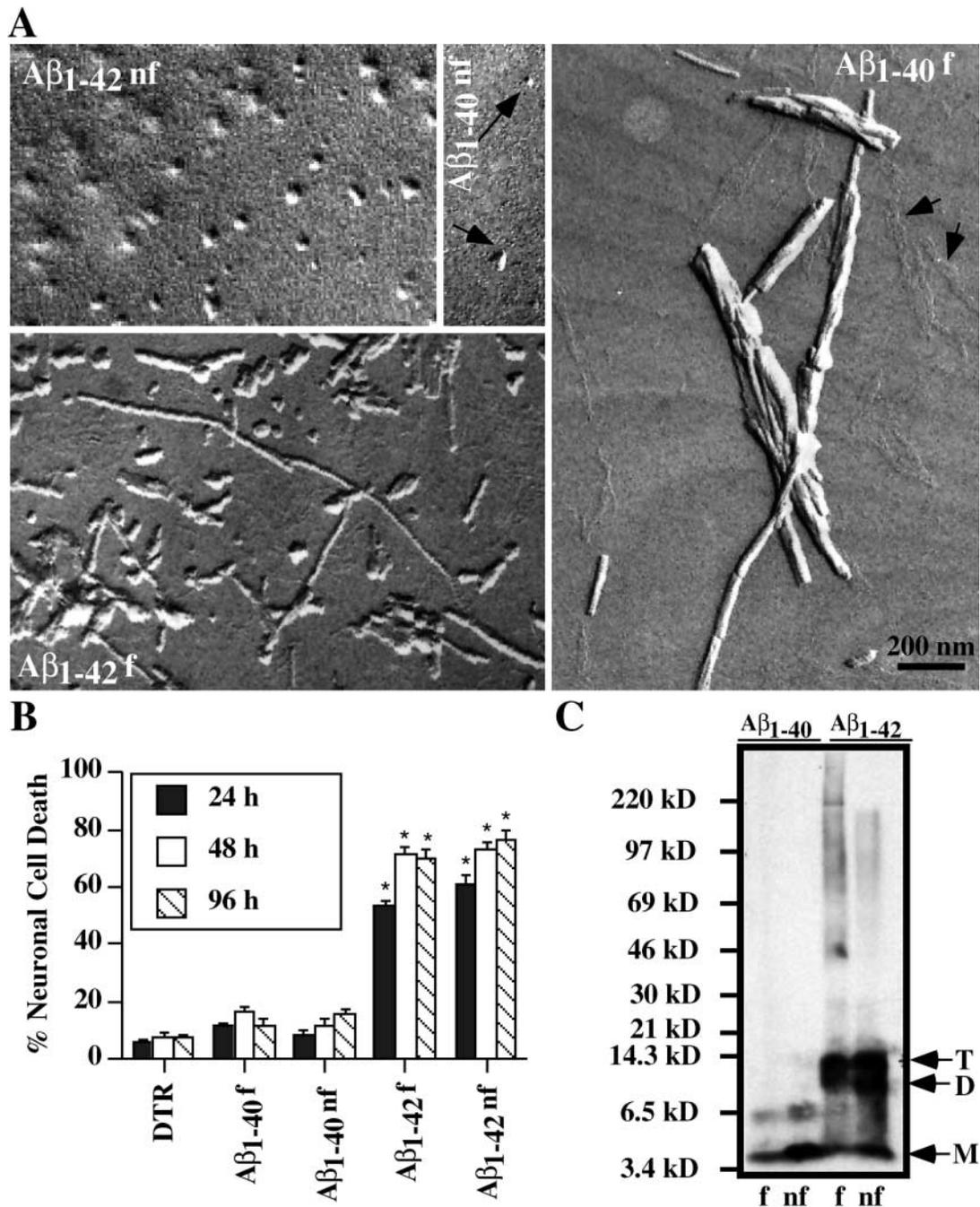
Figure 2. **Soluble and fibrillar Aβ_{1-42} are toxic to human neurons.** (A) Electron micrographs of nonfibrillized (nf) and fibrillized (f) Aβ_{1-40} and Aβ_{1-42}. In the Aβ_{1-40} nf, the arrows point to the rare globular structures while in the Aβ_{1-40} f, the arrows point to the small aligned fibrils. (B) Nonfibrillized or fibrillized Aβ_{1-40} or Aβ_{1-42} (10 nM) were injected into human neurons and neuronal cell death assessed by TUNEL staining at 24, 48, and 96 h after injection. The data represent the mean ± SEM of three independent experiments. One-way ANOVA (df = 14) followed by Sheffé's test were performed to determine the statistical significance between Aβ-injected and control DTR-injected neurons. *, $P < 0.01$. (C) Western blot analysis of fibrillized or nonfibrillized Aβ_{1-40} and Aβ_{1-42} with 6E10. M, D, and T represent the monomeric, dimeric and trimeric forms, respectively. A longer exposure revealed a smear also in the fibrillized Aβ_{1-40} (unpublished data).

Intracellular Aβ_{1-42} toxicity appears selective to human neurons

Because neurons specifically undergo cell death in AD, we explored the specificity of intracellular Aβ toxicity in primary cultured human astrocytes. The volume of these cells is 10 times that of human neurons, therefore, we injected 100 nM of Aβ_{1-42} into the cytosol of astrocytes to keep the same concentration of injected Aβ. 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
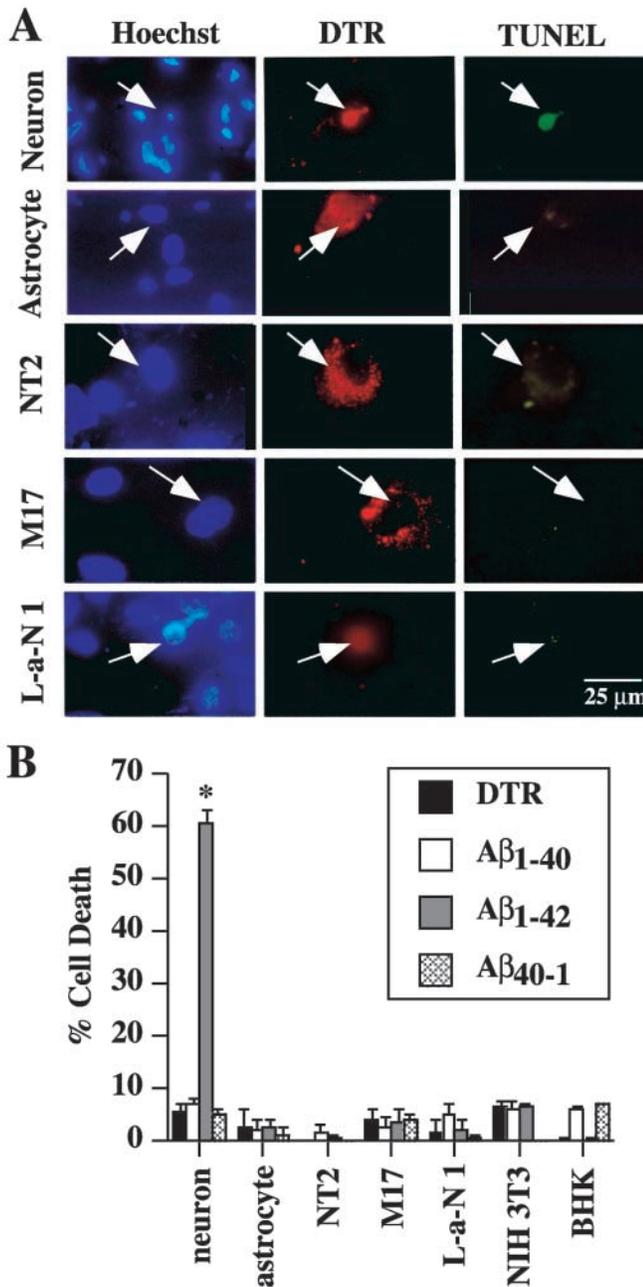
A | Hoechst | DTR | TUNEL (Neuron, Astrocyte, NT2, M17, L-a-N 1)

25 μm

B

Figure 3.   Intracellular Aβ$_{1-42}$ is not toxic to primary human astrocytes, neuroblastoma, teratocarcinoma, fibroblast, and kidney cell lines. (A) Cells were microinjected with 100 nM Aβ$_{1-42}$ and DTR and incubated for 24 h. TUNEL staining identified cell death and Hoechst staining detected nuclei. (B) Cell survival quantitation in several cell lines injected with DTR and Aβ$_{1-40}$, Aβ$_{1-42}$, or Aβ$_{40-1}$ and incubated 24 h. The data represent the mean ± SEM of three independent experiments. One-way ANOVA (df = 27) followed by Sheffé's test determined the statistical significance between Aβ-injected and control DTR-injected cells. *, $P < 0.01$.

red DTR fluorescence on our microscope. Similarly, microinjections of the Aβ$_{1-42}$ 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
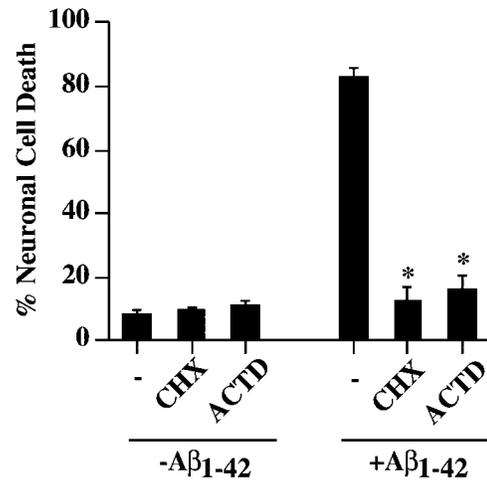

Figure 4.   Intracellular Aβ$_{1-42}$ toxicity requires de novo protein synthesis. Neuronal cell death in non or Aβ$_{1-42}$-injected neurons incubated in the absence (−) or presence of 5 μg/ml cycloheximide (CHX) or 5 μM actinomycin D (ACTD) for 48 h. Neurons were preincubated for 1 h in CHX and ACTD before microinjections. The data represent the mean ± SEM of three independent experiments. One-way ANOVA (df = 5) followed by Sheffé's test determined a statistical significant difference between Aβ$_{1-42}$ in absence and presence of CHX and ACTD but not between CHX and ACTD treatment of non and Aβ$_{1-42}$-injected neurons. *, $P < 0.01$.

lines tested, only human primary neurons are susceptible to the intracellular Aβ$_{1-42}$ 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 Aβ$_{1-42}$ occurs in transgenic animals (LaFerla et al., 1995). Taken together, our data demonstrates that intracellular Aβ$_{1-42}$ is selectively toxic to these human neurons.

Aβ$_{1-42}$ neurotoxicity requires do novo protein synthesis

To address the underlying molecular mechanism of intracellular Aβ toxicity, we microinjected Aβ$_{1-42}$ 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 Aβ$_{1-42}$-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 Aβ$_{1-42}$-mediated cell death. Therefore, we microinjected a human cDNA expression construct of Bcl-2, the major anti-Bax protein, with the Aβ$_{1-42}$ peptide (Fig. 5 A). The microinjection of the Bcl-2 construct completely eliminates Aβ$_{1-42}$-mediated neurotoxicity as observed before against Bax (Bounhar et al., 2001). In contrast, coinjection of an APP cDNA construct does not alter Aβ$_{1-42}$-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
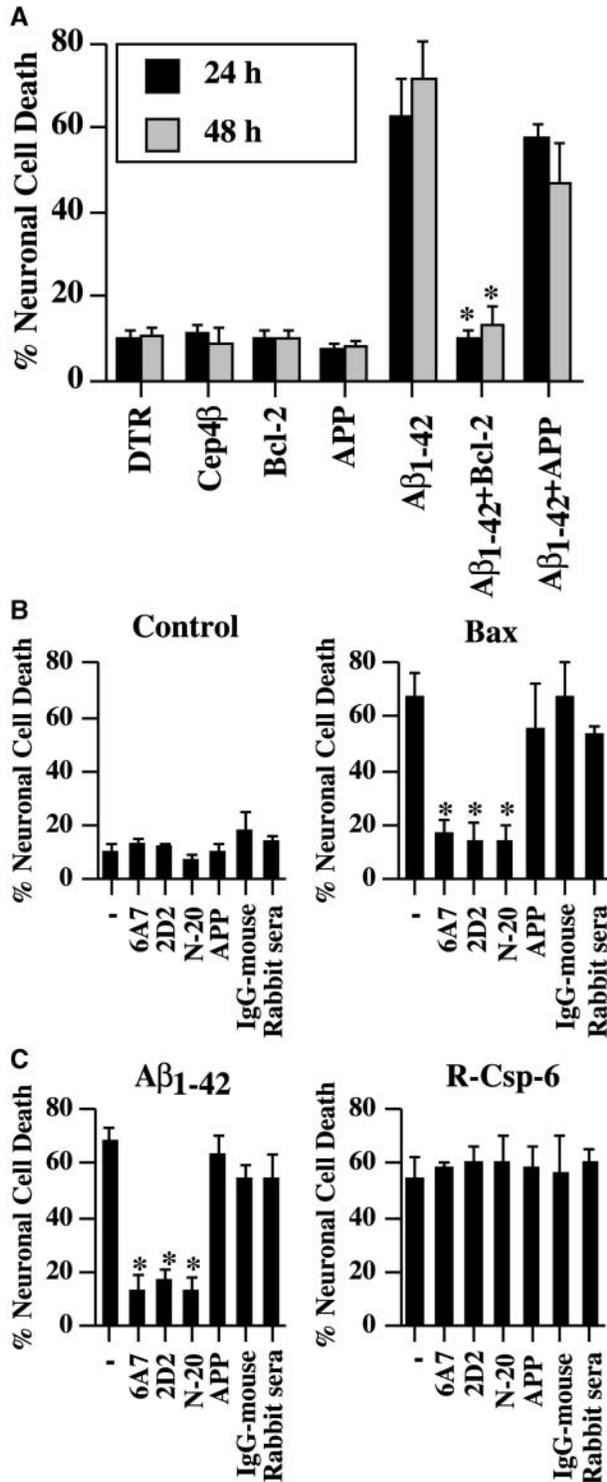
Figure 5. **Inhibition of Aβ_{1-42}-mediated neuronal cell death with Bcl-2 and Bax-neutralizing antibodies.** (A) Cell death in neurons coinjected with Aβ_{1-42} and Bcl-2 or APP pCep4β eukaryotic cDNA expression episomal construct. *$P < 0.01$. (B) Neuronal cell death in DTR alone (control), Bax cDNA, Aβ_{1-42} peptide, or recombinant active caspase-6 (R-Csp-6)-microinjected neurons in the absence (−) or presence of monoclonal Bax antibodies, 6A7 or 2D2, Bax polyclonal antisera, N-20, APP monoclonal antibody 22C11, mouse IgG, or rabbit nonimmune sera. Cells were incubated 24 h after microinjections. The data represent the mean ± SEM of three independent experiments. One-way ANOVA (df = 6) followed by Sheffé's test determined the significance of the difference between the insult in absence and presence of the antibodies. *, $P < 0.01$.
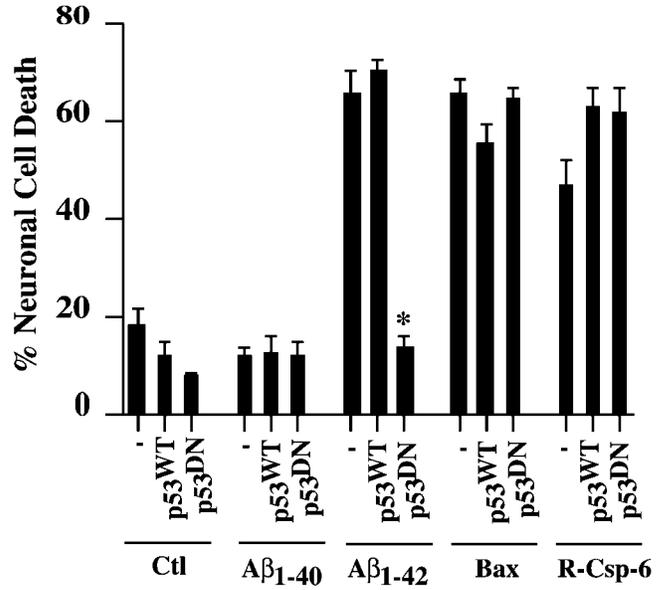

Figure 6. **Inhibition of Aβ_{1-42}-mediated neuronal cell death with p53 dominant negative R273H mutant but not p53 wild type.** Neuronal cell death in DTR (Ctl), Aβ_{1-40} peptide, Aβ_{1-42} peptide, R-Csp-6, or Bax cDNA alone (−) or comicroinjected with cDNA expressing wild-type (WT) or dominant negative (DN) p53 in neurons. Microinjected cells were incubated 48 h. The data represent the mean ± SEM of three independent experiments. One-way ANOVA (df = 14) followed by Sheffé's test showed a statistically significant difference between Aβ_{1-42} without and with p53^{DN}-injected neurons. *, $P < 0.01$.

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 Aβ_{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 Aβ_{1-42}-mediated cell death and indicate that Aβ_{1-42} induces cell death through Bax activation. Together, these results implicate Bax in Aβ_{1-42}-mediated neuronal cell death.

p53 is involved in intracellular Aβ_{1-42}-mediated neurotoxicity

Bax is transcriptionally regulated by p53; therefore, we tested whether p53 activation was involved in intracellular Aβ_{1-42}-mediated neurotoxicity. We chose to use the p53^{R273H} dominant negative (p53^{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^{DN} does not induce neuronal apoptosis in absence or presence of Aβ_{1-40}, the p53^{DN}, but not the p53^{WT}, effectively inhibits Aβ_{1-42}-mediated neurotoxicity (Fig. 6). The inability of the p53^{DN} to inhibit the toxicity of Bax when expressed from the CMV promoter of a cDNA construct or to inhibit cell death by recombinant active caspase-6 attests to the specificity of the p53^{DN} against Aβ_{1-42}-mediated cell death. Together with the inhibition of Aβ_{1-42}-mediated neurotoxicity using Bax antibodies, these results suggest that Aβ_{1-42} activates p53 which regulates Bax expression to induce cell death.

Caspase inhibitors prevent Aβ$_{1-42}$-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 Aβ$_{1-42}$-mediated neurotoxicity, we incubated Aβ$_{1-42}$-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 Aβ$_{1-42}$-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 Aβ$_{1-42}$-induced cell death. Therefore, the data show that caspase-6– or caspase-8–like activities regulate Aβ$_{1-42}$-mediated neuronal apoptosis.

Discussion

The accumulation of intraneuronal Aβ$_{42}$ before the appearance of senile plaques and NFT in AD questions the relevance of intraneuronal Aβ$_{42}$ in neurons (Gouras et al., 2000; D'Andrea et al., 2001). In the present manuscript, we show that (a) very low levels of intracellular Aβ$_{1-42}$, but not Aβ$_{1-40}$, Aβ$_{42-1}$, or Aβ$_{40-1}$, are selectively cytotoxic to human neurons; (b) nonfibrillized Aβ$_{1-42}$ peptide is as toxic as the fibrillized peptide and toxicity may be attributable to the formation of Aβ$_{1-42}$ oligomers; and (c) Aβ$_{1-42}$ toxicity is mediated through the p53 and Bax cell death pathway. Therefore, our findings support the hypothesis that intracellular accumulation of Aβ$_{1-42}$ may be an initial cause of neuronal dysfunction and loss in AD. Intracellular accumulation of Aβ$_{1-42}$ 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 Aβ$_{40}$ and Aβ$_{42}$ 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 Aβ$_{1-42}$ is supported by the results of LaFerla and colleagues (1995) who showed that neurofilament L promoter-directed expression of cytosolic Aβ$_{1-42}$ results in neuronal cell death in mice (LaFerla et al., 1996). Additionally, our experiments show that levels of Aβ$_{1-42}$ likely to be more physiological can rap-
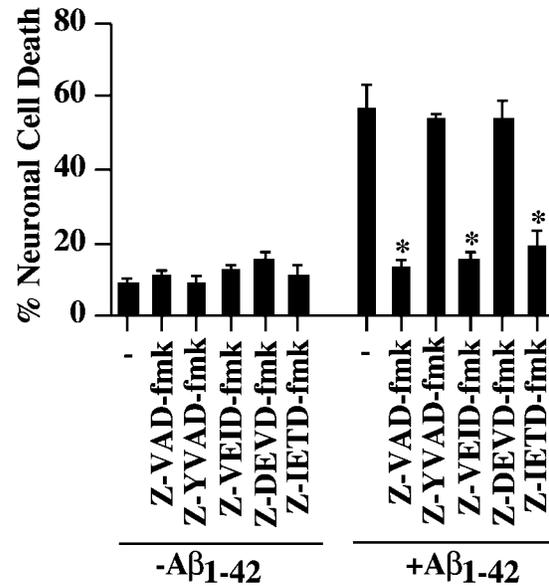

Figure 7. **Inhibition of Aβ$_{1-42}$-mediated neuronal cell death with caspase inhibitors.** Neurons were preincubated for 1 h in the presence of 5 μM of each inhibitor, microinjected with Aβ$_{1-42}$ peptide and incubated for 24 h in the presence of the inhibitors before revealing cell death of injected cells with TUNEL. The data represent the mean ± SEM of three independent experiments. One-way ANOVA (df = 11) followed by Sheffé's test showed a statistically significant difference between Aβ$_{1-42}$ and Aβ$_{1-42}$ + Z-VAD-fmk, Z-VEID-fmk, and Z-IETD-fmk. *, $P < 0.01$.

idly induce cell death and that this toxicity is restricted to a nonfibrillar form of Aβ$_{1-42}$ peptide.

The extreme toxicity of intracellular Aβ$_{1-42}$ raises some important questions. First, can Aβ$_{1-42}$, which is made through the secretory pathway, access the cytosol? Normally, it should not. However, there are three possible modes of entry of Aβ$_{1-42}$ into the cytosol. Insoluble Aβ$_{42}$ 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 Aβ$_{1-42}$ could be compromised by a reduction of proteosomal activity thus resulting in cytosolic Aβ$_{1-42}$ neurotoxicity (Merker et al., 2001). Second, newly synthesized Aβ$_{1-42}$ could be directed to endosomes/lysosomes either from the trans-Golgi or by endocytosis of secreted peptide. In this situation, the Aβ$_{1-42}$ 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 Aβ$_{1-42}$, but not Aβ$_{1-40}$, increases lysosomal membrane permeability possibly resulting in leakage of the Aβ$_{1-42}$ in the cytosol (Yang et al., 1998). Third, the secreted Aβ$_{1-42}$ 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 Aβ$_{1-42}$ or Aβ$_{1-40}$ 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 $A\beta$ receptors or endocytosis or alters the plasma membrane permeability resulting in the diffusion of the extracellular $A\beta_{1-42}$ peptide inside the cell. However, this latter possibility is unlikely to be a primary cause of AD, as intracellular $A\beta_{1-42}$ 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 $A\beta_{1-42}$ compared with $A\beta_{1-40}$ poses another interesting problem: the extracellular $A\beta$ peptide toxicity has been attributed to the fibrillar, protofibrillar, or aggregating properties of $A\beta$ (for review see Klein et al., 2001). For intracellular $A\beta_{1-42}$ toxicity, there exists three possibilities. First, it could be that the extra two amino acids on $A\beta_{1-42}$ are responsible for inducing toxicity. Second, as has previously been suggested, amyloid fibrils could be the toxic molecules. However, both less toxic $A\beta_{1-40}$ and extremely toxic $A\beta_{1-42}$ form significant amounts of fibrils and nonfibrillized $A\beta_{1-42}$ is still very toxic. Third, the oligomers observed by Western blot analysis in the nonfibrillized and fibrillized $A\beta_{1-42}$ 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 change when microinjected in neurons due to association with certain cytosolic factors (Walsh et al., 1999; Nilsberth et al., 2001). Therefore, much work will be required to elucidate the toxic structure of the $A\beta_{1-42}$ peptides.

We further show that $A\beta_{1-42}$ mediates neurotoxicity through the known p53 and Bax cell death pathway. p53 expression increases in the transgenic neurons of cytosolically expressed $A\beta_{1-42}$ and in AD neurons (LaFerla et al., 1996; de la Monte et al., 1997, 1998; Kitamura et al., 1997; Seidl et al., 1999). In addition, synthetic p53 inhibitors can prevent extracellular $A\beta$ mediated toxicity of hippocampal neuron cultures (Culmsee et al., 2001). Extracellular $A\beta$ can upregulate Bax expression or require Bax to mediate cytotoxicity (Paradis et al., 1996; Selznick et al., 2000; Culmsee et al., 2001). Furthermore, Bax protein levels increase in AD (MacGibbon et al., 1997; Nagy and Esiri, 1997; Su et al., 1997; Tortosa et al., 1998; Giannakopoulos et al., 1999), although this was not confirmed by all studies (Engidawork et al., 2001). Therefore, the identification of the role of the p53–Bax cell death pathway in intracellular $A\beta_{1-42}$-mediated neurotoxicity reveals additional therapeutic targets that could be used against AD. Because p53 is activated through phosphorylation, the results suggest that $A\beta_{1-42}$ induces a kinase or inhibits a phosphatase responsible for this phosphorylation. Given the known importance of tau hyperphosphorylation in AD, one wonders whether the activation of p53 could be linked to the phosphorylation of tau through intracellular $A\beta_{1-42}$ induction of kinase activity.

The mechanism by which p53 is activated by intracellular $A\beta_{1-42}$ remains to be elucidated. It is interesting to note that $A\beta_{1-42}$ interacts with ERAB, an intracellular ER and mitochondria-localized member of the alcohol dehydrogenase family (Yan et al., 1997). In transfected Cos cells, ERAB facilitates the toxicity of extracellular $A\beta_{1-42}$ and APP^{V717G}

mutant (Yan et al., 1999). Whether ERAB is involved in cytosolic $A\beta_{1-42}$ toxicity remains to be determined but since ERAB is localized to mitochondria and ER, it is unlikely to have access to the injected $A\beta_{1-42}$ unless mitochondrial ERAB is released into the cytosol during apoptosis as are many other mitochondrial factors.

It is well known that Bax activates caspases by promoting the release of mitochondrial cytochrome c, which forms an apoptosome with a number of other factors including caspases (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 $A\beta_{1-42}$ 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 $A\beta_{1-42}$.

Uncovering the acute human neuronal toxicity of intracellular $A\beta_{1-42}$ questions the validity of the currently developed therapies against extracellular amyloid in AD. If intracellular $A\beta_{1-42}$ 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 $A\beta_{1-42}$ 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 $A\beta_{1-42}$ should also have a favorable impact on the disease assuming that it is only $A\beta_{1-42}$ 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 caspases 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). APP$_{695}$ cDNA in pCep4β was obtained from Steve Younkin (Mayo Clinic, Jacksonville, FL). The cDNAs were cloned into pCep4β (Invitrogen). The p53 wild-type and p53^{R273H} 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^{TM} (GIBCO BRL) and diluted at 30 ng/μl in PBS before the microinjections.

Primers were designed to amplify secreted and cytosolic $A\beta_{1-40}$ and $A\beta_{1-42}$ from APP$_{695}$. These primers amplify the entire $A\beta$ sequence with an additional methionine ATG codon at the 5' end and a stop codon at the 3' end to ensure translation. Cytosolic $A\beta$ was amplified with the $A\beta_{1-40/42}$ forward primer 5'-TCA CTC GAG AAT GGA TGC AGA ATT CCG ACA T-3' (contains a built-in 5' XhoI site) and $A\beta_{1-42}$ reverse primer 5'-ATG

GAT CCT TAC GCT ATG ACA ACA CCG AA-3' (has a 3' BamH1 site) or Aβ$_{1-40}$ reverse primer 5'-TCG ATC CTT AGA CAA CAC CGC CCA CCA TG-3' (has a 3' BamH1 site). Secreted Aβ was made by linking the APP signal peptide (SP) to the Aβ sequence. The SP was amplified with APP-SP1 forward primer, 5'-TTA CTC GAG ATG CTG CCC GGT TTG GCA-3' (contains a 5' XhoI site) and APP-SP2 reverse primer, 5'-GGA ATT CTG CAT CCA TCG CCC GAG CCG TCC AGG C-3' (contains a 3' EcoR1 site). A ligation between EcoR1- cleaved PCR-amplified Aβ coding sequence and the EcoR1-cleaved SP sequence was reamplified with the Aβ$_{1-40/42}$ forward primer and Aβ$_{1-40}$ or Aβ$_{1-42}$ reverse primers. The PCR-amplified SP and Aβ sequence were cloned into the prokaryotic pBSKII and eukaryotic episomal pCep4β vectors through the XhoI/BamHI restriction sites. All clones were restriction mapped and sequenced before use.

Neutralizing Bax 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 rabbit sera were diluted at 50 µg/ml (for 6A7, 2D2, mouse IgG, and 22C11) or 25 µg/ml (for polyclonal anti-Bax and rabbit sera) in PBS before use. A toxicity curve was done to determine these concentrations as the highest nontoxic concentrations that can be injected in neurons.

Cell cultures
Primary cultures of human neurons and astrocytes were prepared from 11–17-wk-old fetal brains as described previously (LeBlanc, 1995). The McGill University Institutional Review Board (Montréal, Québec, Canada) has approved this procedure following Canadian Institutes of Health and National Institutes of Health ethical guidelines. Brain tissues were dissociated with 0.25% trypsin (GIBCO BRL) in PBS at 37°C for 15 min. The trypsin was inactivated with 10% decomplemented FBS (HyClone). The dissociated cells were triturated in 0.1 mg/ml DNaseI (GIBCO BRL), filtered successively through 130 µm nylon mesh (Sefar Canada, Inc.) and Falcon 70-µm cell strainers (Becton Dickinson), and centrifuged at 5,000 g for 10 min at 10°C to pellet the cells. The cell pellet was washed once with PBS and once with MEM (GIBCO BRL) in Earle's balanced salt solution containing 0.225% sodium bicarbonate, 1 mM sodium pyruvate, 2 mM L-glutamine, 0.1% dextrose, antibiotic Pen-Strep (all from GIBCO BRL), and 5% decomplemented FBS. The cells were plated at a density of 3×10^6 cells/ml on poly-L-lysine–coated ACLAR™ (Cat. No., 33C; thickness, 0.5 mm; Allied Chemical) coverslips. Neuron cultures were treated successively 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 (Cellular Biochemistry and Genetics, New York, NY) and cultured on ACLAR™ coverslips at 10^6 cells/ml in OPTI-MEM (GIBCO BRL) containing 5% FBS. 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 10^6 cells/ml in DME (GIBCO BRL) containing 10% FBS. BHK cells, a gift from William Bowers (University of Rochester, Rochester, NY), and the mice NIH3T3 fibroblasts, a gift from Dr. Stephane Richard (McGill University, Montréal, Québec, Canada) were grown in DME and 10% FBS.

Aβ peptides
Initially (for Fig. 1), Aβ 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 Aβ 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 solution was incubated at 37°C in Eppendorf tubes with continuous mixing by inversion to fibrillize the peptides. After incubation, the samples were removed, vortexed, sonicated twice for 1 min in a bath type sonicator (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 Aβ peptide was placed on freshly cleaved mica plates (BioForce Laboratory, Inc.). The specimens were air dried and subsequently transferred to a Balzers High-Vacuum Freeze-Etch Unit (model 301) under a 1.3×10^{-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-mesh grid (Canemco, Inc.). The grids were examined with a Joel 200FX transmission electron microscope (Joel) at 21,000× magnification.

Western blot analysis of Aβ peptides
Nonfibrillar and fibrillar forms of Aβ$_{1-40}$ and Aβ$_{1-42}$ (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 milliamps for 2 h. The membrane was blocked by 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-Aβ$_{1-17}$ antibody 6E10 (Signet) and detected by chemiluminescence.

Microinjection
Thin-walled Borosilicate glass capillaries (OD 1.0 mm, ID 0.5 mm) with microfilament (MTW100F-4; World Precision Instrument) were pulled 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 Microinjector 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-Tyrosine-Valine-Alanine-Aspartic acid-fmk (Z-YVAD-fmk), caspase-6 inhibitor, Z-Valine-Glutamic acid-Isoleucine-Aspartic acid-fmk (Z-VEID-fmk), caspase-3 inhibitor, Z-Aspartic acid-Glutamic acid-Valine-Aspartic acid-fmk (Z-DEVD-fmk), and caspase-8 inhibitor, Z-Isoleucine-Glutamic acid-Threomine-Aspartic acid-fmk (Z-IETD-fmk) (Sigma-Aldrich), were dissolved in 100% DMSO (Sigma-Aldrich) at 20 mM and were 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 (ANOVAs) with post hoc tests (Statview 5.01) determined the statistical significance of the difference between treatments. The Dunnett's test was used when comparing several groups 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-

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

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