

# Amyloid $\beta$ Peptide of Alzheimer's Disease Downregulates Bcl-2 and Upregulates Bax Expression in Human Neurons

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Neuronal apoptosis is a suspected cause of neurodegeneration in Alzheimer's disease (AD). Increased levels of amyloid  $\beta$  peptide ( $A\beta$ ) induce neuronal apoptosis *in vitro* and *in vivo*. The underlying molecular mechanism of  $A\beta$  neurotoxicity is not clear. The normal concentration of  $A\beta$  in cerebrospinal fluid is 4 nM. We treated human neuron primary cultures with 100 nM amyloid  $\beta$  peptides  $A\beta_{1-40}$  and  $A\beta_{1-42}$  and the control reverse peptide  $A\beta_{40-1}$ . We find that although little neuronal apoptosis is induced by either peptide after 3 d of treatment,  $A\beta_{1-42}$  provokes a rapid and sustained downregulation of a key anti-apoptotic protein, bcl-2, whereas it increases levels of bax, a protein known to promote cell death. In contrast, the  $A\beta_{1-40}$  downregulation of bcl-2 is gradual, although the levels are equivalent to those of  $A\beta_{1-42}$ -

42-treated neurons by 72 hr of treatment.  $A\beta_{1-40}$  does not upregulate bax levels. The control, reverse peptide  $A\beta_{40-1}$ , does not affect either bcl-2 or bax protein levels. In addition, we found that the  $A\beta_{1-40}$ - and  $A\beta_{1-42}$ - but not  $A\beta_{40-1}$ -treated neurons had increased vulnerability to low levels of oxidative stress. Therefore, we propose that although high physiological amounts of  $A\beta$  are not sufficient to induce apoptosis,  $A\beta$  depletes the neurons of one of its anti-apoptotic mechanisms. We hypothesize that increased  $A\beta$  in individuals renders the neurons vulnerable to age-dependent stress and neurodegeneration.

**Key words:** amyloid  $\beta$  peptide; human neuron primary cultures; bcl-2; bax; apoptosis; Alzheimer's disease

Genetic, molecular, and cell biology evidence support a role for the amyloid  $\beta$  peptide ( $A\beta$ ) of 40 and 42 amino acids ( $A\beta_{1-40}$  and  $A\beta_{1-42}$ ) in the pathogenesis of Alzheimer's disease (AD): (1) Mutations of the amyloid precursor protein (APP), which is normally processed into  $A\beta$  and a number of stable fragments, increase the production of  $A\beta_{1-40}$  or  $A\beta_{1-42}$  (Citron et al., 1992, 1994; Cai et al., 1993; Haass et al., 1994; Suzuki et al., 1994); (2) mutations of presenilin I and II genes of familial AD increase APP mRNA and  $A\beta$  levels (Levy-Lahad et al., 1995; Querfurt et al., 1995; Sherrington et al., 1995; Sheuner et al., 1996); (3) the ratio of  $A\beta_{1-42}$  to  $A\beta_{1-40}$  increases in sporadic AD brains (Iwatsubo et al., 1994; Kuo et al., 1996); (4) Down's syndrome individuals who carry three copies of the APP gene and consequently overexpress APP mRNA and produce excess  $A\beta$  invariably develop AD pathology (Wisniewski et al., 1985; Tanzi et al., 1987; Teller et al., 1996); and (5) transgenic mice overexpressing normal APP, mutant APP<sub>717</sub>, or  $A\beta$  develop features of AD pathology (Games et al., 1995; Higgins et al., 1995; Hsiao et al., 1995; LaFerla et al., 1995).

$A\beta$ s cause rat hippocampal and cortical neuronal death *in vitro* (concentrations of 1–100  $\mu$ M) and *in vivo* (Yankner et al., 1990; LaFerla et al., 1995). Treatment of cultured neurons with 25–100  $\mu$ M  $A\beta$  induces typical features of apoptosis: neurite beading, membrane blebbing, condensed chromatin, and DNA

fragmentation (Forloni et al., 1993; Loo et al., 1993; Watt et al., 1994). Evidence of neuronal apoptosis is also observed in sporadic and familial AD brains (Anderson et al., 1994, 1996; Su et al., 1994; Satou et al., 1995; Yamatsuji et al., 1996). The mechanism by which  $A\beta$  induces cell death or apoptosis is not yet clearly defined.  $A\beta$  alters calcium levels, thereby promoting susceptibility to excitotoxic damage (Mattson et al., 1992), promotes free radical formation (Behl et al., 1994; Shearman et al., 1994), and possibly competes for nerve growth factor low-affinity receptor (Rabizadeh et al., 1994). In glial cells,  $A\beta$  induces the production of cytokines, tumor necrosis factor, and reactive nitrogen intermediates, any of which can mediate neuronal cell death (Meda et al., 1995). Furthermore,  $A\beta$  induces hyperphosphorylation of tau, which may promote neurofibrillary tangle formation and neurodegeneration (Busciglio et al., 1995).

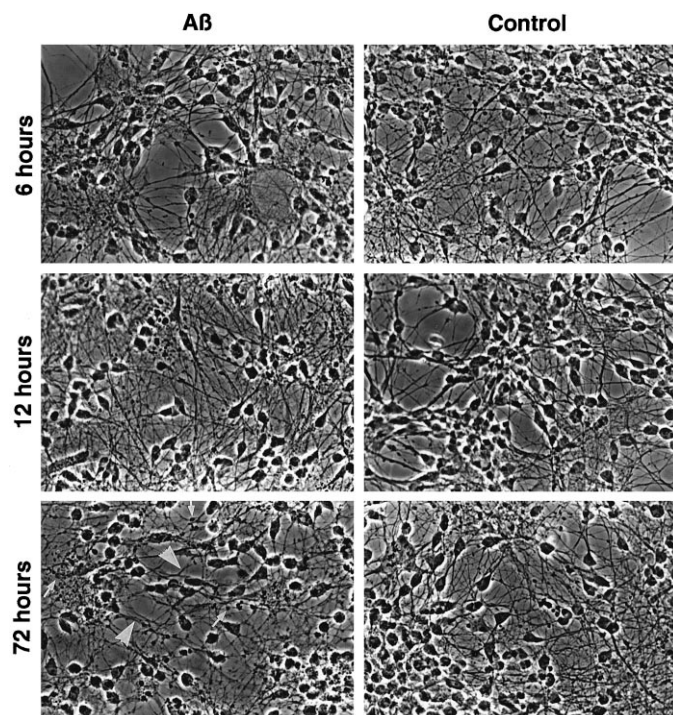
In the present study, we analyzed the effect of 100 nM  $A\beta_{1-40}$  and  $A\beta_{1-42}$  and control reverse peptide  $A\beta_{40-1}$  on human primary neuron cultures. We measured cell death by various techniques and examined the effect of  $A\beta$  on neuronal "death" bax and "anti-death" bcl-2 proteins. We do not detect significant cell death with  $A\beta_{1-40}$ ,  $A\beta_{1-42}$ , or  $A\beta_{40-1}$ , although there is a slight increase in terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) after 72 hr of treatment with  $A\beta_{1-40}$  and  $A\beta_{1-42}$  compared with  $A\beta_{40-1}$ . Interestingly,  $A\beta_{1-40}$  and  $A\beta_{1-42}$  but not  $A\beta_{40-1}$  downregulate bcl-2. Furthermore,  $A\beta_{1-42}$  upregulates bax. In addition, the  $A\beta$ -treated neurons show increased vulnerability to low levels of oxidative stress. We hypothesize that  $A\beta$  jeopardizes the natural bcl-2-related protective mechanism in neurons and that this effect may lead to increased neuronal degeneration during age-dependent stresses such as oxidative

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**Figure 1.** Neurite degeneration is observed after 72 hr of treatment with  $A\beta$  peptides. Phase-contrast micrograph of primary neuron cultures treated with 100 nM  $A\beta_{1-40}$  or  $A\beta_{1-42}$  and control reverse peptide  $A\beta_{40-1}$  for 6, 12, and 72 hr. Arrows show neuritic beading, and arrowheads show neurite thickening.

stress, decreased energy metabolism, or decreasing growth factor levels.

## MATERIALS AND METHODS

**Neuron cultures.** Human primary neurons were prepared as described (LeBlanc, 1995) from fetal brains of 13–17 weeks (Munsick, 1984). The brains were collected from human fetuses at the time of the therapeutic abortion in accordance with the Quebec Health Code (received Institutional Review Board approval). Briefly, the cerebrum is dissociated in trypsin (Life Technologies, Ontario) and deoxyribonuclease I (Boehringer Mannheim, Quebec), filtered through 40 and 130  $\mu$ m nylon mesh, and plated at  $3 \times 10^6$  cells/ml of high-glucose-containing MEM supplemented with Earle's salts, 1 mM sodium pyruvate, 2 mM glutamine (all from Life Technologies), and 5% decomplexed serum (Hyclone, Logan, UT); 1 mM anti-mitotic fluorodeoxyuridine (Sigma, St. Louis, MO) is added to inhibit proliferation of contaminating astrocytes or other dividing cells. Under these conditions, neurons establish healthy neurite networks within 3 d and can be maintained for at least 4 weeks without showing signs of degeneration. Experiments were carried out on neurons 10 d after plating. The neuronal cultures typically contain >90% neurons.

**Treatment of neurons with  $A\beta$ .** Fibrillar  $A\beta_{1-40}$ ,  $A\beta_{1-42}$  (Bachem Bioscience, Torrance, CA), and control reverse peptide  $A\beta_{40-1}$  (Sigma) were prepared by incubating freshly solubilized peptides at 25  $\mu$ M in sterile distilled water at 37°C for 5 d (Pike et al., 1993). At the beginning of each experiment,  $A\beta$  peptides were diluted to 100 nM in complete media. Neurons were treated for 6, 12, 24, 48, and 72 hr. For the 3 d incubation, the neuron media was changed after 48 hr.

**Western blot analysis of bcl-2 and bax.** After  $A\beta$  peptide treatment on  $3 \times 10^6$  cells, neurons were washed in PBS and collected in 300  $\mu$ l NP-40 lysis buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 1% NP40, 5 mM EDTA, pH 8.0) containing 0.05% phenylmethylsulfonyl fluoride, 0.1  $\mu$ g/ml pepstatin A, 1  $\mu$ g/ml  $N\alpha$ -p-tosyl-L-lysine chloromethyl ketone, and 0.5  $\mu$ g/ml leupeptin as protease inhibitors (all protease inhibitors from ICN, Montreal). Seventeen microliters of the lysate were submitted to electrophoresis on a 10% SDS-polyacrylamide gel and Western blot analysis with monoclonal anti-human bcl-2, sc-509 (Santa Cruz Biotech, Santa

Cruz, CA), polyclonal anti-human bax, sc-493 (Santa Cruz Biotech), or monoclonal anti-human actin (kind gift from Dr. Eugenia Wang, Lady Davis Institute and McGill University). Immunoreactivity was detected using secondary anti-mouse (for bcl-2 and actin) or rabbit (for bax) antibodies conjugated to alkaline phosphatase (Jackson ImmunoResearch Laboratories, West Grove, PA) and substrates nitroblue tetrazolium and 5-bromo-chloro-3-indoyl-phosphate (Fisher, Montreal). The resulting Western blots were scanned on a Molecular Dynamics phosphorimager densitometric scanner. The data (pixels) were expressed as standard units, where 1 unit = bcl-2 or bax levels in 0 hr untreated neuron cultures. Statistical significance was determined by a two-tailed unpaired *t* test of  $A\beta_{1-40}$  or  $A\beta_{1-42}$  values compared with those of  $A\beta_{40-1}$ .

**MTT and lactate dehydrogenase (LDH) assays.** Analysis of released LDH was performed by ELISA using the Cytotoxic 96 kit according to the manufacturer's instructions (Pharmacia Biotech, Quebec). The MTT reduction assay was carried out using the Cell Proliferation Kit I (Boehringer Mannheim). The MTT assay measures both cell death and proliferation and is based on the conversion of the yellow tetrazolium salt MTT to blue formazan by metabolically active cells. The resulting blue color was measured at absorbance 660nm and 550 nm. MTT reduction was calculated as  $Abs_{660nm} - Abs_{550nm}$ , and results are expressed as a percentage of the control untreated sample. Increased absorbance readings with time indicate proliferation, whereas decreased absorbance indicates cell death.

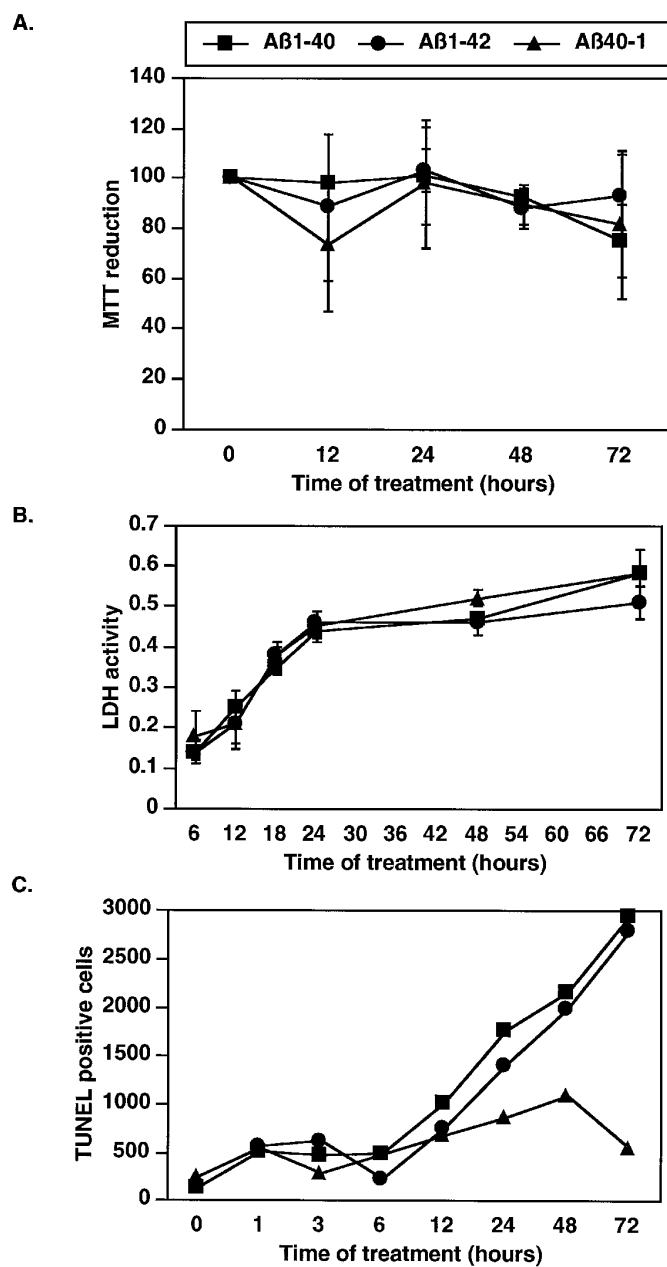
**Determination of apoptosis in cells.** Neurons were fixed in fresh 4% paraformaldehyde for 20 min and permeabilized in 0.1% Triton X-100, 0.1% sodium citrate. Cells were stained with 0.1  $\mu$ g/ml propidium iodide (Sigma) for 20 min, rinsed in PBS, and mounted. TUNEL was performed using the *in situ* cell death detection kit AP as described by the manufacturer (Boehringer Mannheim, Quebec). DNA ladder was performed on  $3 \times 10^6$  cells. The cells were washed and harvested in PBS. After centrifugation, the cell pellet was incubated in 50  $\mu$ l of a proteinase K/sarkosyl solution (50 mM Tris, pH 8.0, 10 mM EDTA, 0.5 mg/ml proteinase K, and 0.5% sarkosyl) for 2 hr at 50°C; 2.5  $\mu$ l of RNaseA at 10 mg/ml was added, and the incubation continued for 2 hr at 50°C. The DNA was loaded on a 1.2% agarose gel in  $1 \times$  TBE and run at 50 V overnight.

**Oxidative stress on neurons preexposed to  $A\beta$ .** Neurons were treated for 48 hr with  $A\beta_{1-40}$ ,  $A\beta_{1-42}$ , and  $A\beta_{40-1}$  as described above. The media was replaced with 0, 0.1, and 1.0  $\mu$ M  $H_2O_2$  and incubated for another 48 hr. Cells were fixed and processed for TUNEL as described above. Because the number of apoptotic cells was very high in  $H_2O_2$ -treated neurons, five specific areas of each coverslip were counted and added as number of TUNEL-positive cells. The density of neurons required for survival in culture does not allow determination of exact cell numbers in this experiment. Therefore the results are expressed as total number of TUNEL-positive cells rather than percentage.

## RESULTS

### Neuronal degeneration in $A\beta$ -treated neurons

To study the effect of the  $A\beta$  peptides, 10-d-old human primary neurons with elaborate neurite networks were treated with 100 nM fibrillar  $A\beta_{1-40}$  and  $A\beta_{1-42}$  for 6, 12, 18, 24, 48, and 72 hr.  $A\beta_{1-40}$  at 100 nM is  $\sim 25 \times$  that of physiological  $A\beta$  concentrations in cerebrospinal fluid (CSF) (Nakamura et al., 1994; Van Gool et al., 1994). Physiological concentrations of  $A\beta_{1-42}$  are not known. Our neuron cultures produce  $\sim 1/10$   $A\beta_{1-42}$  compared with  $A\beta_{1-40}$  (A. LeBlanc and S. Younkin, unpublished results). Therefore, 100 nM  $A\beta_{1-42}$  likely represents 250  $\times$  the physiological concentration in CSF. Phase-contrast micrography of acid/alcohol-fixed neurons shows that neurons do not undergo neurodegeneration in an acute manner with either  $A\beta_{1-40}$  or  $A\beta_{1-42}$  (Fig. 1). At 72 hr of treatment, neurites are thickened and beaded compared with control  $A\beta_{40-1}$ -treated cells, but neither the cell density nor the neuronal bodies seem to be affected. The neuritic alterations in  $A\beta$ -treated human fetal neurons is variable with each culture, suggesting that the heterogeneity of the sample population results in different degrees of susceptibility to  $A\beta$  peptides. In



**Figure 2.**  $A\beta$  peptides do not cause high levels of cell death in neuron primary cultures. Determination of cell death in  $A\beta_{1-40}$ ,  $A\beta_{1-42}$ , and  $A\beta_{40-1}$ -treated human primary neurons for 12, 24, 48, and 72 hr by MTT reduction assay expressed as a percentage of time 0 ( $n = 3 \pm$  SEM) (A), LDH release assay ( $n = 3 \pm$  SEM) (B), and TUNEL-positive cells in a representative experiment (C).

time, however, each neuron culture treated with either  $A\beta_{1-40}$  or  $A\beta_{1-42}$  undergoes neurite beading by 72 hr of treatment.

#### Studies of cell death in human primary neurons treated with $A\beta_{1-40}$ , $A\beta_{1-42}$ , and $A\beta_{40-1}$

Cell death in culture is generally measured by the reduction of MTT activity and the release of LDH into the media. MTT is a tetrazolium salt cleaved to formazan by the mitochondrial respiratory chain enzyme succinate-tetrazolium reductase, which is active only in live cells. LDH is released into the cell media when cells die by necrosis. Although slightly decreasing

MTT levels and increasing LDH activity with time indicate a certain amount of cell death in our cultures, there is no significant difference between  $A\beta_{1-40}$ ,  $A\beta_{1-42}$ , and  $A\beta_{40-1}$ -treated neurons (Fig. 2A,B). To determine whether apoptotic cell death occurred in the neuronal cultures, we looked for fragmented DNA by TUNEL staining, condensed chromatin by propidium iodide staining, and DNA ladder formation as a function of time of treatment. Although few condensed chromatin-containing neurons were observed by propidium iodide staining of three independent experiments (results not shown),  $\sim 2\%$  of the neurons treated with  $A\beta_{1-40}$  and  $A\beta_{1-42}$  for 72 hr were TUNEL-positive against a background of 0.5% in  $A\beta_{40-1}$ -treated neurons (Fig. 2C). The absence of DNA ladder formation confirmed the low level of apoptotic neurons in the  $A\beta$ -treated cultures (not shown). These results were reproduced in three independent experiments.

#### $A\beta$ downregulates bcl-2 and upregulates bax

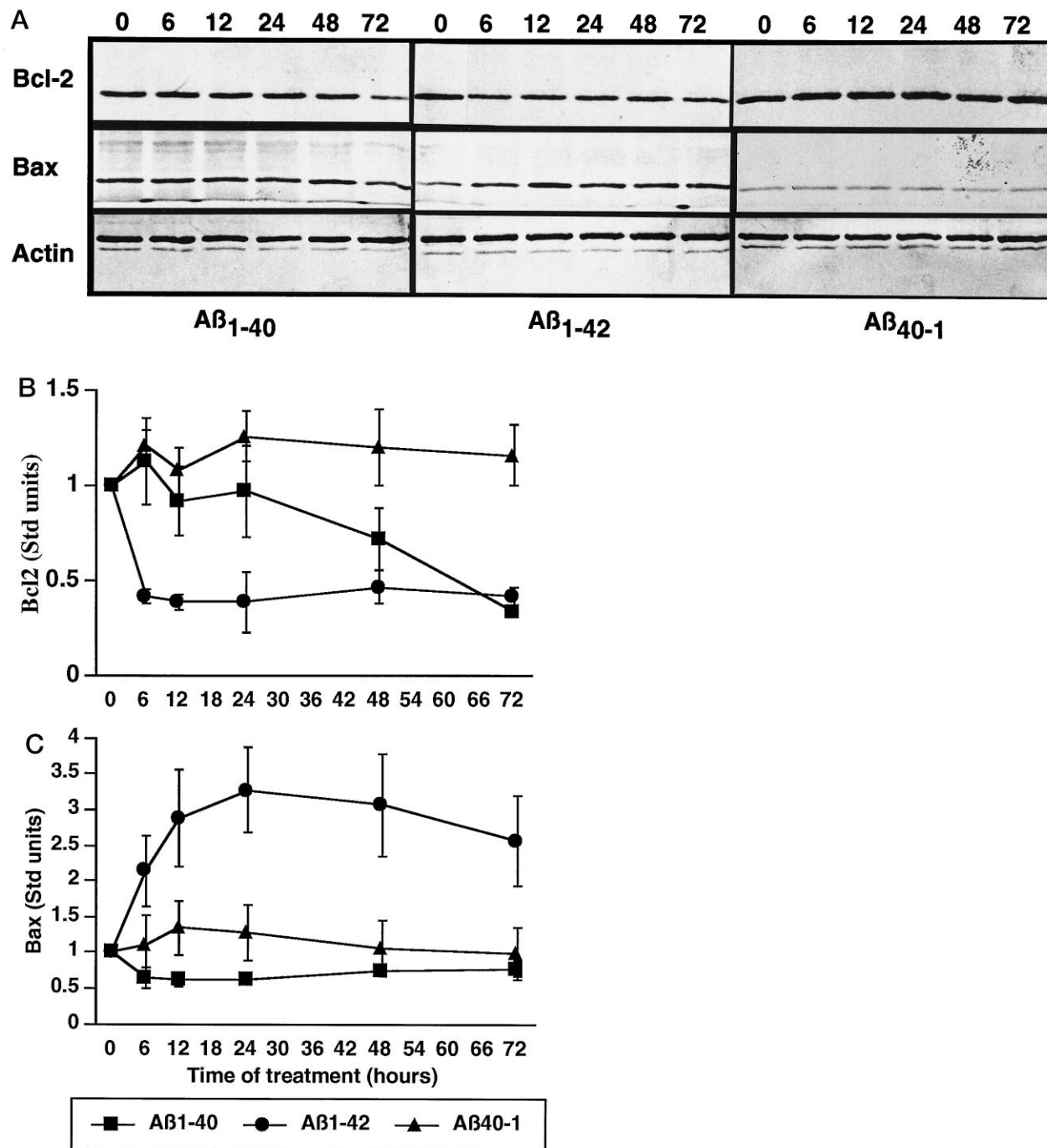
Bcl-2 and bax levels were assessed by Western blots to determine whether these key regulators of apoptosis were involved in the  $A\beta$ -induced neurotoxic mechanism (Fig. 3). Using a monoclonal anti-human bcl-2 antibody and a polyclonal antisera to bax, we consistently saw decreased bcl-2 protein levels in  $A\beta_{1-40}$ - and  $A\beta_{1-42}$ -treated neurons compared with  $A\beta_{40-1}$  control cells (Fig. 3A). With  $A\beta_{1-42}$ , we also observed increased bax protein levels. The equivalent levels of actin detected from each time point attest to the equal protein loading in each well of the gel. We quantitated the decreased bcl-2 and increased bax protein levels by densitometric scanning with a phosphorimager. We find that the bcl-2 protein level normalized to untreated cultures (0 hr) is rapidly reduced by  $>50\%$  in  $A\beta_{1-42}$ -treated neurons compared with  $A\beta_{40-1}$ -treated cells (Fig. 3B). The effect is observed within 6 hr of treatment and sustained for up to 72 hr.  $A\beta_{1-40}$  also downregulates bcl-2 protein levels, but the effect is gradual, and a significant difference with the control  $A\beta_{40-1}$  reverse peptide is observed only at 72 hr.

In contrast to bcl-2 protein levels, bax protein increases three- to fourfold in  $A\beta_{1-42}$ -treated neurons compared with  $A\beta_{40-1}$  (Fig. 3C). The increase in bax is apparent at 6 hr and peaks at 24 hr. The  $A\beta_{1-40}$  has no effect on bax levels in neurons.

The results indicate that a 25-fold increase in the level of  $A\beta_{1-40}$  and a 250-fold increase in  $A\beta_{1-42}$  are not sufficient to induce significant apoptosis, although some cells in the neuronal culture are clearly more vulnerable than others to the peptides. Downregulation of bcl-2 and upregulation of bax in these  $A\beta$ -treated neurons demonstrate that the peptides have an effect on proteins that mediate apoptosis. To determine whether the neurons exposed to  $A\beta$  were more vulnerable to an age-dependent secondary insult such as oxidative stress, we first treated the neurons with  $A\beta_{1-40}$  and  $A\beta_{1-42}$  for 48 hr and then exposed them to low oxidative stress levels, using 0.1 and 1.0  $\mu\text{M}$   $\text{H}_2\text{O}_2$ . The  $A\beta_{1-40}$ - and  $A\beta_{1-42}$ -treated cultures contain 10–20 times more TUNEL-positive cells when treated with 0.1 and 1.0  $\mu\text{M}$   $\text{H}_2\text{O}_2$ , whereas these low levels of  $\text{H}_2\text{O}_2$  do not increase apoptosis in  $A\beta_{40-1}$ -treated neurons (Fig. 4). These levels of oxidative stress also had no effect on untreated neurons that showed results equivalent to those of the control  $A\beta_{40-1}$  (not shown).

#### DISCUSSION

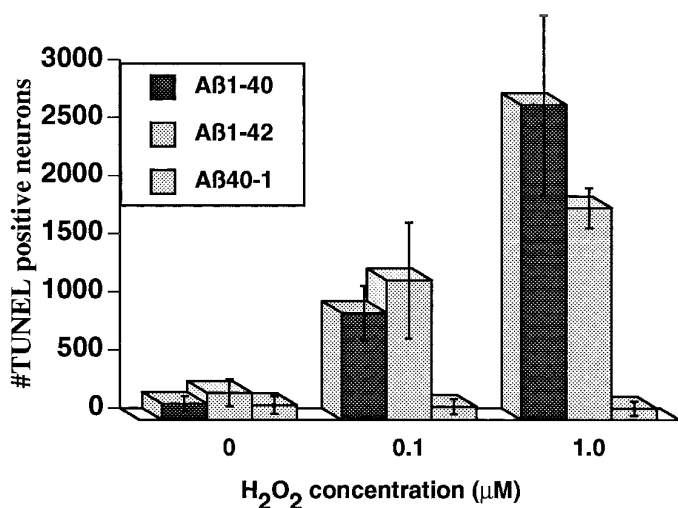
We find that elevated but physiological concentrations of  $A\beta_{1-40}$  and  $A\beta_{1-42}$  are insufficient to initiate significant human



**Figure 3.**  $A\beta$  peptides downregulate bcl-2 protein and upregulate bax protein levels in primary neuron cultures. *A*, Western blot analysis of one representative experiment of bcl-2, bax, and actin protein levels in neurons treated with  $A\beta_{1-40}$ ,  $A\beta_{1-42}$ , and  $A\beta_{40-1}$  for 0, 6, 12, 24, 48, and 72 hr. *B*, Densitometric quantitation by phosphorimager of bcl-2 protein levels in neuron cultures normalized to levels in 0 hr culture (mean of three independent experiments,  $\pm$  SEM) shows rapidly decreasing bcl-2 protein levels in  $A\beta_{1-42}$  ( $p < 0.01$ , from 6–72 hr) and a gradual decrease in  $A\beta_{1-40}$ -treated neurons ( $p < 0.01$ , at 72 hr only) compared with  $A\beta_{40-1}$ -treated neurons. *C*, Densitometric quantitation by phosphorimager of bax protein levels in neuron cultures normalized to levels in untreated (0 hr) sister culture (mean of three independent experiments,  $\pm$  SEM) shows rapidly increasing bax protein levels in  $A\beta_{1-42}$  ( $p < 0.05$ , between 24–72 hr) but no difference in  $A\beta_{1-40}$ - compared with  $A\beta_{40-1}$ -treated neurons.

neuronal apoptosis in cultures. Nanomolar concentrations of fresh or aged  $A\beta$  that are likely to be physiological concentrations do not show neurotoxic effects in most systems, and human neurons have previously been shown to be resistant to micromolar concentrations of  $A\beta$  (Mattson et al., 1992; Shear-

man et al., 1994). Yet, there is considerable evidence that  $A\beta$  plays a primary role in Alzheimer's disease. We find that 100 nM  $A\beta_{1-42}$  rapidly decreases bcl-2 protein levels in neurons, whereas it increases bax levels. Interestingly, bcl-2 protein levels are also decreased in 100 nM  $A\beta_{1-40}$ -treated neurons, but



**Figure 4.** Susceptibility to H<sub>2</sub>O<sub>2</sub> in neurons pretreated with Aβ<sub>1-40</sub> and Aβ<sub>1-42</sub> but not Aβ<sub>40-1</sub>. TUNEL labeling detects increased apoptosis in neurons pretreated for 48 hr with Aβ<sub>1-40</sub> and Aβ<sub>1-42</sub> and then submitted to 0.1 μM H<sub>2</sub>O<sub>2</sub> and 1.0 μM H<sub>2</sub>O<sub>2</sub> for 48 hr. In contrast, sister cultures treated with the control peptide Aβ<sub>40-1</sub> do not show increased sensitivity to low levels of oxidative stress. Data represent an average of four experiments.

only gradually, and reach Aβ<sub>1-42</sub>-treated levels by 72 hr, whereas bax levels remain unaffected. In addition, neurons preexposed to either Aβ<sub>1-40</sub> or Aβ<sub>1-42</sub> show increased sensitivity to a mild oxidative stress. Therefore, we propose a novel hypothesis that in Alzheimer's disease, neuronal degeneration may be caused by Aβ-mediated downregulation of bcl-2 rendering the neurons vulnerable to age-dependent secondary insults such as oxidative stress, decreasing levels of growth factors, or diminished glucose metabolism (Coyle and Puttfarcken, 1993).

Aβ neurotoxicity has been widely investigated in a number of neuronal systems with the use of very high concentrations of the aggregated or fibrillar peptide (1–100 μM). The underlying mechanisms mediating Aβ neurotoxicity are attributed to either oxidative stress (Behl et al., 1994; Thomas et al., 1996) or the destabilization of intracellular calcium levels (Mattson et al., 1992). Our observations are consistent with both mechanisms. Bcl-2 is well established as an anti-death protein in neurons. Bcl-2 can avert survival factor deprivation-induced neuronal apoptosis in sympathetic cervical ganglia, in sensory primary neurons, and in continuous cell lines such as PC12 cells (Garcia et al., 1992; Batistatou et al., 1993; Mah et al., 1993; Martinou et al., 1994). Bcl-2 and another neuronal bcl-2-related anti-death protein, bcl-xL, also prevent hypoxia and axotomy-induced neuronal death *in vivo* (Dubois-Dauphin et al., 1994; Jacobson and Raff, 1995; Shimizu et al., 1995). Therefore, bcl-2 and related anti-death proteins are key regulators of programmed cell death in neurons. Any disruption in the level of these proteins is likely to result in increased neuronal vulnerability. The exact mechanism by which downregulation of bcl-2 enhances neuronal vulnerability to oxidative stress is not clear. Although it has been proposed by some that bcl-2 directly protects cells against reactive oxygen species (Hockenbery et al., 1993; Kane et al., 1993; Veis et al., 1993), others find that bcl-2 protects cells without decreasing reactive oxygen species (Jacobson and Raff, 1995; Shimizu et al., 1995).

On the other hand, it has been shown that in human neurons Aβ enhances excitotoxicity, and this effect is attributed to increased intracellular calcium (Mattson et al., 1992). Increased calcium levels are also linked to apoptosis, and it has been suggested that bcl-2 is involved in the maintenance of cellular calcium homeostasis (Bafy et al., 1993). Downregulation of bcl-2 in human neurons may explain the dysregulation of intracellular calcium levels by Aβ. Our results show that the effect of Aβ<sub>1-42</sub> on bcl-2 and bax levels is more drastic than that of Aβ<sub>1-40</sub>. These results are consistent with the higher aggregative potential of Aβ<sub>1-42</sub> (Jarrett et al., 1993) and the aggregation-dependent neurotoxicity of Aβ peptides (Pike et al., 1993).

On the basis of the low levels of neurotoxicity in our human primary cultures exposed to high physiological concentrations of Aβ and the fact that familial AD is manifestly a disease of the aged, we propose that Aβ by itself is unlikely to induce apoptosis in AD patients and that it requires a second age-dependent factor to be neurotoxic. Our data suggest the possibility that downregulation of bcl-2 in the presence of Aβ renders the cell vulnerable to age-dependent stress. Although there is no overwhelming evidence for apoptotic neurons in AD brains, there are indications that such a mechanism can occur. Bcl-2 immunoreactivity generally is increased in AD neurons but decreased in degenerating neurons (Satou et al., 1995). Other evidence of apoptosis in AD brains includes increased apopTag and c-jun immunoreactivity, evidence of DNA fragmentation, and bcl-2-sensitive apoptosis induced by familial AD mutants of APP (Mullaart et al., 1990; Anderson et al., 1994; Su et al., 1994; Lassmann et al., 1995; Anderson et al., 1996; Yamatsuji et al., 1996). The effect of Aβ on fetal neurons may be criticized as a model for AD, an aging disease. The fact remains that these are the only source of human neurons at this time. The decreased bcl-2 expression observed in degenerating neurons of AD brains (Satou et al., 1995) supports the possibility that the effect of Aβ on bcl-2 levels in fetal neuron cultures is reproduced *in vivo* in the brain of adult individuals.

In summary, our data show that although 25-fold Aβ<sub>1-40</sub> and 250-fold Aβ<sub>1-42</sub> concentrations do not cause extensive apoptosis in human neurons, Aβ does alter the anti-apoptotic protective balance by downregulating bcl-2 and upregulating bax protein levels, and it increases the vulnerability of these cells to oxidative stress. The data support the hypothesis that increased Aβ concentrations in AD CSF render the neurons vulnerable to age-dependent stresses such as oxidative stress.

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