Amyloid-β Peptides Are Cytotoxic to Oligodendrocytes

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Alzheimer's disease (AD) is a neurodegenerative disease characterized by progressive dementia. Amyloid-β peptide (Aβ), a 39–43 amino acid peptide derived from β-amyloid precursor protein, forms insoluble fibrillar aggregates that have been linked to neuronal and vascular degeneration in AD and cerebral amyloid angiopathy. Here we demonstrate that Aβ 1–40 and a truncated fragment, Aβ 25–35, induced death of oligodendrocytes (OLGs) *in vitro* in a dose-dependent manner with similar potencies. Aβ-induced OLG death was accompanied by nuclear DNA fragmentation, mitochondrial dysfunction, and cytoskeletal disintegration. Aβ activation of redox-sensitive transcription factors NF-κB and AP-1 and antioxidant prevention of Aβ-mediated OLG death suggest that oxidative injury contributes to Aβ cytotoxicity in OLGs. Recent demonstration of Aβ deposition and white matter abnormalities in AD implies a potential pathophysiological role for Aβ-mediated cytotoxicity of OLGs in this neurodegenerative disease.

Key words: Alzheimer's disease; apoptosis; cell death; mitochondrial DNA; oxidative stress; white matter

The pathological hallmarks of Alzheimer's disease (AD) include neuritic and cerebrovascular plaques containing amyloid-β peptide (Aβ), neurofibrillary tangles, activated glia, and neuronal degeneration (Selkoe, 1999). Aβ, a 39–43 amino acid fragment derived from β-amyloid precursor protein (βAPP), forms insoluble fibrillar aggregates that have been linked to neuronal and vascular degeneration in AD brains (Masters et al., 1985; Yankner et al., 1989; Thomas et al., 1996). Although Aβ has been shown to be cytotoxic to neurons (Yankner et al., 1989; Behl et al., 1994) and endothelial cells (Thomas et al., 1996), the effect of Aβ toxicity on oligodendrocytes (OLGs) has not been studied. In human brains, Aβ deposits have been noted in close proximity to damaged OLGs in both white and gray matter (Yamada et al., 1997). Aβ can activate OLGs to produce chemokines (Johnstone et al., 1999) as well as microglia and astrocytes to synthesize inflammatory mediators, including cytokines iNOS and COX2 (Meda et al., 1995; Akama et al., 1998; Griffin et al., 1998; McGeer and McGeer, 1999). Microglia have been shown to process Aβ (Chung et al., 1999). These observations suggest that glia, as potential inflammatory cells, may respond differently than neurons to Aβ.

Although most attention has focused on the extensive gray matter pathology in AD, there is growing recognition that white matter is also commonly affected. White matter damage is readily demonstrated by magnetic resonance imaging studies (Scheltens et al., 1992; O'Brien et al., 1996), and extent of this damage is associated with dementia severity (Stout et al., 1996). Neurophysiological studies based on delayed latencies in visual and brainstem auditory evoked potentials raise the possibility of myelin dysfunction in AD brains (Tanaka et al., 1998). AD pathology in white matter includes loss of myelin and axons (Brun and Englund, 1986), as well as OLG loss and DNA fragmentation (Brun and Englund, 1986; Lassmann et al., 1995). A high percentage of AD patients show evidence of white matter degeneration or leukoaraiosis with severe loss of OLGs caused by apoptosis (see Brown et al., 2000).

The observed AD pathology in white matter might be an indirect consequence of neuronal damage in gray matter, or of white matter vascular insufficiency (Brun and Englund, 1986). Alternatively, such damage could be mediated directly by Aβ deposition, which has been reported in white matter in human AD (Wisniewski et al., 1989) and in animal models of AD (Holtzman et al., 2000). In this study, we used an enriched OLG culture to examine whether OLGs are directly vulnerable to Aβ cytotoxicity. We studied the effect of Aβ 1–40 and Aβ 25–35 on OLG viability; Aβ 25–35 is a truncated sequence of Aβ 1–40. Aβ 25–35 also forms fibrils and is cytotoxic to neurons by a mechanism similar to that of Aβ 1–40/42 (Behl et al., 1994). We characterized selected aspects of death mechanism in Aβ-induced OLG cytotoxicity. Results strongly suggest that Aβ-induced oxidative injury is a plausible mechanism of OLG death. Characterization of Aβ toxicity to OLGs and the death mechanism involved may increase our understanding of its potential role in white matter lesions in AD.

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MATERIALS AND METHODS

OLG culture. OLGs cultures were prepared as described previously (Levison and McCarthy, 1991; McDonald et al., 1998) with modifications. Briefly, 1- to 2-d-old rat brain cortex was loosely homogenized in DMEM with 10% serum, filtered (80 μm nylon mesh), and centrifuged at 1000 rpm for 10 min. The cells were grown in 75 mm flasks (1.5 brains per flask) for 7–10 d until confluent. The flasks were agitated at 180 rpm at 37°C for 1.5 hr to remove microglia and then for another 18 hr to harvest OLGs. The suspension containing OLGs was filtered through 10 μm nylon mesh, resuspended in a chemically defined medium [CDM/DMEM/F-12 (1:1)], and plated onto 100 mm dishes, 24-well plates, or coverslips. Characteristic morphology and the expression of OLG-specific markers including galactocerebroside (GalC), Rip, and cyclic nucleotide 3′-phosphodiesterase (CNPase) indicated minimally 85% purity. Experiments were performed on OLGs grown for 3–5 d.

OLG death assessment. OLG viability was quantitated by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay (Xu et al., 1998). The cytosolic levels of histone-associated DNA fragments were determined with a Cell Death Detection ELISA kit (Boehringer Mannheim, Indianapolis, IN), and Aβ-induced OLG DNA damage was assessed by DNA laddering and terminal deoxynucleotidyl transferase-mediated biotinylated dUTP nick end labeling (TUNEL) using procedures described previously (Xu et al., 1998).

Immunocytochemistry. Cells were identified by immunostaining for OLG-specific markers including GalC, Rip, and CNPase. GalC and cytochrome *c* immunocytochemistry were used to characterize Aβ-induced OLG death. Aβ-treated OLGs on coverslips fixed with 4% paraformaldehyde were incubated with anti-GalC (1:100; Boehringer Mannheim) or anti-cytochrome *c* antibody (1:200; PharMingen, San Diego, CA) and rhodamine-conjugated goat anti-mouse IgG (1:100). Secondary antibody alone was used as negative control. Nuclei were visualized with 1 μg/ml 4′, 6-diamidino-2-phenylindole dihydrochloride (DAPI) (Molecular Probes, Eugene, OR).

Western blotting. The cytosolic protein fraction was isolated and immunoblotted as described previously (Xu et al., 2000) using a primary monoclonal anti-cytochrome *c* antibody (1:1000; PharMingen) and followed by a secondary alkaline phosphatase-conjugated goat anti-mouse IgG antibody (1:5000; Promega, Madison, WI).

Long PCR for mitochondria DNA. Total DNA was isolated from rat OLGs using a DNA isolation kit (Qiagen, Chatsworth, CA), and the DNA concentration was quantitated by the Pico Green method (Molecular Probes). The extent of mitochondria (mt)DNA damage was assessed by a long PCR method (Barnes, 1994). The 10 μl PCR reaction mixture contained 0.4 ng total OLG DNA, 4 pmol oligonucleotide primer pair, 400 μM dNTP mix, and 0.5 U LA *Taq* (Takara Shuzo, Madison, WI). As an internal standard, equal concentrations of mouse brain DNA were added to each reaction. The primers used to amplify the 14.3 kb rat and mouse mtDNA were 5′-ATATTTATCACTGCTGAGTCCCGTGG-3′ and 5′-AATTTCGGTTGGGGTGACCTCGGAG-3′. Samples were initially denatured for 1 min at 94°C and amplified for 26 cycles consisting of denaturation at 94°C for 15 sec with primer annealing/extension at 68°C for 10 min. The final extension was at 72°C for 10 min. The PCR condition described above was within the linear portion of the curves for both number of cycles and total DNA input. The long PCR products were treated with *Nco*I (Promega) at 37°C for 2 hr to cleave specifically the product derived from the mouse mtDNA into 7.0 and 7.3 kb fragments. The 14.3 kb rat long PCR product was separated from the smaller 7.0/7.3 kb mouse entities by 1% agarose gel electrophoresis. Ethidium bromide delineated bands were quantitated by a Phosphorimager (Molecular Dynamics, Sunnyvale, CA). mtDNA damage was measured by changes in quantity in the rat PCR product relative to that of mouse.

Alkaline gel electrophoresis and Southern blotting. Total OLG DNA (6 μg) was digested with *Sac*II (Promega) to linearize mtDNA. DNA samples were extracted with phenol/chloroform, precipitated with ethanol, and quantitated. A 5 μg sample was electrophoresed on a 1% agarose gel at 0.5 V/cm in 30 mM NaOH and 2 mM EDTA buffer for 24 hr. After neutralization, DNA was transferred onto Hybond N+ nylon membrane, prehybridized for 2 hr, and hybridized overnight at 60°C with a dig-labeled 413-bp PCR product (primer: 5′-TAGAATGAATGGCTAAACGAGG–3′ and 5′-TTAATAGCTTCTGCACCATTGG–3′; Dig Probe Synthesis Kit, Boehringer Mannheim) complementary to the 16S rRNA sequence in mtDNA.

Electrophoretic mobility shift assay. Crude nuclear extracts from OLGs were prepared as described previously, and electrophoretic mobility shift assay (EMSA) was performed with NF-κB (5′-AGTTGAGGGGACTTTCCCAGGC-3′) and AP-1 (5′-CGCTTGATGAGTCAGCCGGAA-3′) consensus oligonucleotides (Promega) end-labeled with [γ-^{32}P] ATP (An et al., 1993). The binding reaction was performed in 20 μl of 10 mM Tris-HCl, 20 mM NaCl, 1 mM DTT, 1 mM EDTA, 5% glycerol, pH 7.6, containing 15 μg nuclear protein, 0.0175 pmol labeled probe (>30,000 cpm), and 1 μg poly(dI-dC). After incubation at 25°C for 20 min, the reaction mixture was electrophoresed on a nondenaturing 6% polyacrylamide gel at 180 V for 2 hr under low ionic strength.

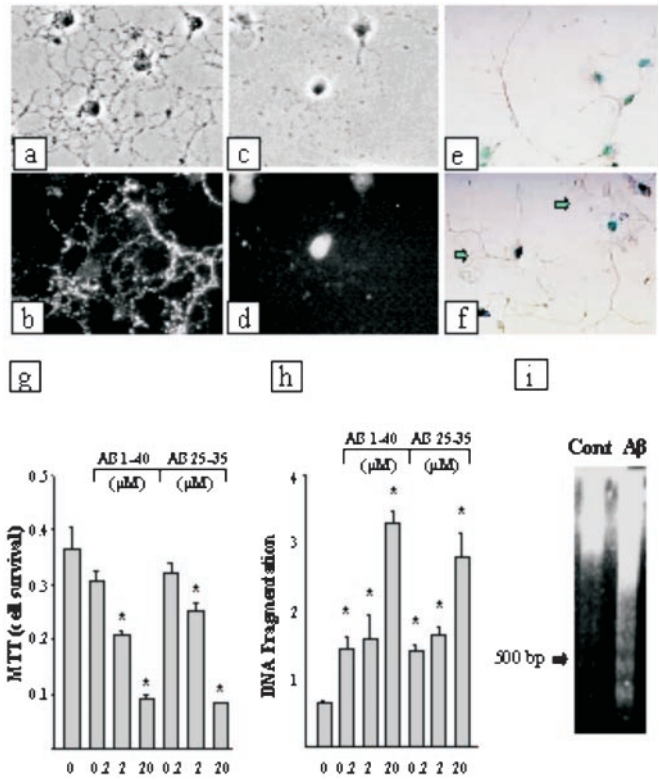

Figure 1. Aβ-induced morphological changes and cytotoxicity in OLGs. *a*, Light microscopy of normal OLG with characteristic branching cellular processes and (*b*) fluorescent microscopy demonstrating intense GalC immunoreactivity in normal OLGs. *c*, Disintegration of the cellular processes and shrinkage of cell bodies and (*d*) loss of GalC immunoreactivity after 20 μM Aβ 25–35 exposure for 24 hr. *e*, *f*, TUNEL stain of OLGs without and with 20 μM Aβ 25–35 treatment for 24 hr. *Arrows* mark TUNEL(+) cells. *g*, MTT assay measuring cell survival. *h*, Nuclear DNA damage based on ELISA measurement of cytosolic content of histone-associated DNA strand breaks. Data shown were from three separate experiments in quadruplicates. * denotes a significant difference from the controls ($p < 0.05$). *i*, DNA laddering in Aβ-treated OLGs (20 μM Aβ 25–35 for 24 hr).

Statistical analyses. Quantitative data are expressed as mean ± SD based on two or three separate experiments in triplicate or quadruplicate. Difference among groups was statistically analyzed by one-way ANOVA followed by Bonferroni's *post hoc* test. Comparison between two experimental groups was based on two-tailed *t* test. A p value <0.05 was considered significant.

RESULTS

Aβ-induced OLG death

Cultured OLGs were readily distinguished under light as small, round, phase-dark cell bodies with branched processes (Fig. 1*a*). Fluorescent microscopy shows that OLGs in culture express characteristic cell markers including, GalC (Fig. 1*b*), Rip, and CNPase (data not shown). Treatment with 20 μM Aβ25–35 resulted in the breakdown and dissolution of OLG processes and appearance of shrunken cell bodies. These morphological changes were detectable 24 hr after Aβ exposure (Fig. 1*c*) concomitant with the loss of GalC immunoreactivity (Fig. 1*d*). Aβ

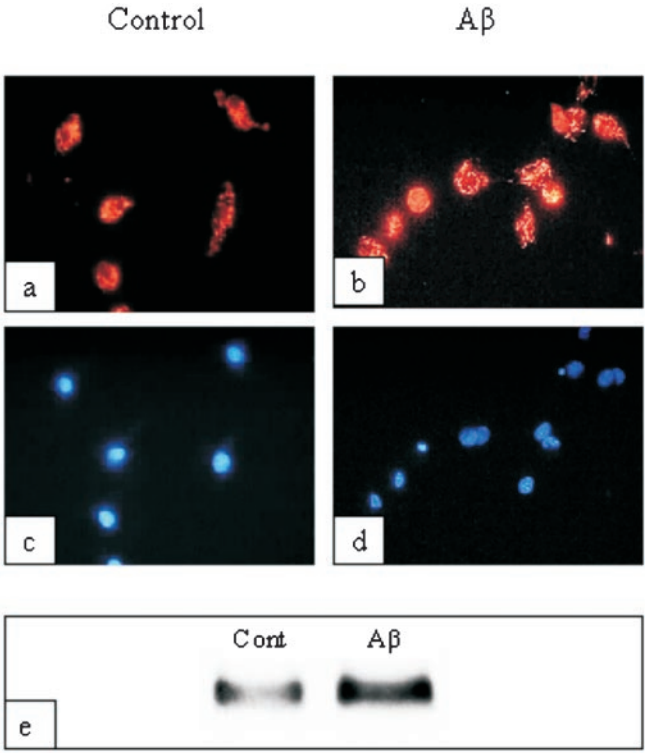

Figure 2. Aβ-induced cytochrome *c* release and nuclear condensation. OLGs were treated with 20 μM Aβ 25–35 for 24 hr. Immunostaining for cytochrome *c* showed (*a*) control OLGs with punctate versus (*b*) uniform cytosolic distribution in Aβ-treated cells. In the same field, DAPI staining shows (*c*) normal nuclei in controls versus (*d*) nuclear condensation in the same Aβ-treated cells with cytochrome *c* release shown in *b*. *e*, Western blotting confirmed Aβ-induced cytochrome *c* release.

1–40 caused similar morphological changes in OLGs (data not shown). TUNEL(+) cells were noted in OLGs treated with 20 μM Aβ 25–35 (Fig. 1*f*) but not in controls (Fig. 1*e*). The MTT assay showed that treatment with 0.2–20 μM Aβ 1–40 or 0.2–20 μM Aβ 25–35 resulted in OLG death in a dose-dependent manner (Fig. 1*g*). Aβ 1–40 and 25–35 were equally toxic, with 20 μM of either peptide causing approximately 75% cell death. Aβ cytotoxicity was also noted in OLGs derived from embryonic preprogenitor cells with >98% purity as assessed by GalC, Rip, and CNPase immunoreactivity (our unpublished observations). An ELISA that measured the cytosolic content of DNA strand breaks was also used to quantitate cell death. Aβ 1–40 or Aβ 25–35 (20 μM) increased the cellular mononucleosome and oligonucleosome levels by four- to fivefold over controls (Fig. 1 *h*). Aβ-induced DNA damage was further confirmed by DNA laddering on agarose gel electrophoresis (Fig. 1*i*).

Aβ-induced mitochondrial dysfunction and oxidative stress in OLGs

Aβ-induced OLG death was associated with extensive DNA damage supportive of an apoptotic cell death mechanism. Mitochondria have been implicated as the center of execution in apoptotic cells (Green and Reed, 1998). A key feature of apoptosis involving mitochondria is the release of cytochrome *c*. Immunocytochemical studies showed mitochondrial cytochrome *c* redistribution in OLGs treated with 20 μM Aβ 25–35 (Fig. 2*a*,*b*). DAPI counterstaining of the same microscopic fields showed condensed nuclei in the OLGs with cytochrome *c* redistribution

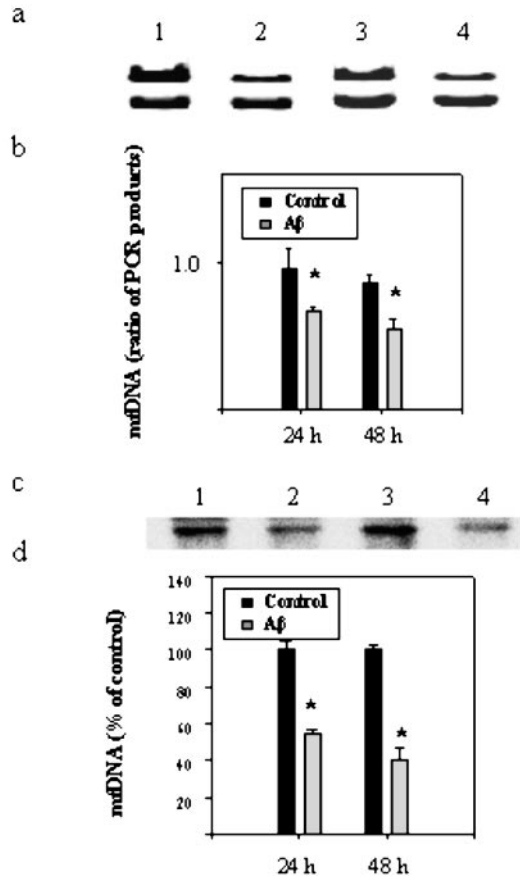

Figure 3. Aβ-induced oxidative stress in OLGs. *a*, Aβ 25–35 (20 μM) induced mtDNA damage shown by the long PCR method. *Top bands* are rat OLG mtDNA; *bottom bands* are mouse brain mtDNA internal standards. *c*, Alkaline gel electrophoresis confirming mtDNA damage. *b* and *d* represent quantitation of the PCR and Southern blot data, respectively. * denotes that difference from control is significant.

suggestive of apoptosis (Fig. 2*c*,*d*). Aβ-induced OLG cytochrome *c* release into cytosol was confirmed by Western blotting (Fig. 2*e*).

Aβ also caused mtDNA damage in OLGs as demonstrated by a long PCR method (Fig. 3*a*) and Southern blot alkaline gel electrophoresis (Fig. 3*c*). Similar levels of Aβ-induced mtDNA damage were detected in OLG cells derived from embryonic preprogenitor cells with >98% purity (our unpublished observations). Aβ treatment caused a significant reduction in mtDNA content (Fig. 3*b*,*d*). Aβ-induced mtDNA damage is indicative of increased oxidative stress (Bozner et al., 1997). Another consequence of oxidative stress is the activation of transcription factors, such as NF-κB and AP-1, that are sensitive to the redox state (Abate et al., 1990; Schreck et al., 1991; Pinkus et al., 1996). Aβ increased NF-κB and AP-1 binding activity in OLGs (Fig. 4*a*). These results suggest that Aβ-induced OLG death was accompanied by an enhanced oxidative state. *N*-acetylcysteine (NAC), a potent antioxidant that has been shown to reduce Aβ-mediated neuronal death (Behl et al., 1994), protected OLGs against Aβ 25–35 cytotoxicity (Fig. 4*b*).

DISCUSSION

In this study, we demonstrated that both Aβ 1–40 and its truncated fragment, Aβ 25–35, induced OLG death in culture. Cytotoxic effects of Aβ 1–40 and Aβ 25–35 were dose-dependent and have equal potencies, with 20 μM of each peptide causing 75% cell

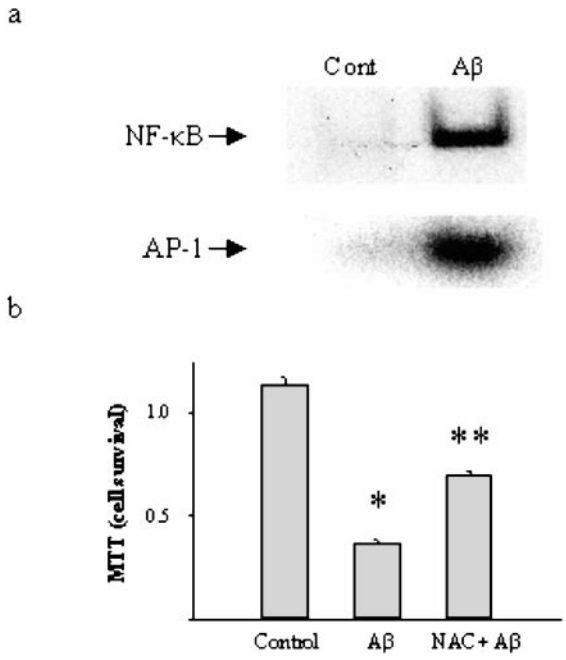

Figure 4. Oxidative stress in Aβ-treated OLGs. *a*, EMSA showing NF-κB and AP-1 activation after 20 μM Aβ 25–35 treatment for 24 hr. *b*, NAC effect on Aβ-induced OLG death. Untreated OLGs or OLGs treated with 5 mM NAC for 2 hr were incubated with 10 μM Aβ 25–35 for 24 hr, and viability was measured by MTT assay. Data were from two separate experiments in triplicate. * denotes differences from control; ** denotes differences from Aβ treatment alone are significant.

death. Decreased OLG viability was accompanied by nuclear chromatin condensation, DNA fragmentation and laddering, and cytochrome *c* release. These findings are compatible with OLG death by an apoptotic mechanism. Aβ-treated OLGs also showed morphological features suggestive of diffuse destruction of cytoarchitecture extending from the nucleus to cytoplasmic processes. Thus OLG death entails a mixed death mechanism encompassing both apoptosis and necrosis. Free radicals have been shown to cause both necrosis and apoptosis.

Aβ-induced oxidative stress is a prominent feature in Aβ-mediated neuronal death (Behl et al., 1994; Behl, 1999; Markesbery, 1999). The extensive cell damage in Aβ-induced OLG death including nucleus and cytoplasmic processes is compatible with that caused by oxygen free radicals. An increase in oxidative stress induces mtDNA damage (Bozner et al., 1997) and activates selected transcription factors such as NF-κB and AP-1 (Abate et al., 1990; Schreck et al., 1991; Pinkus et al., 1996). We noted that Aβ treatment did induce mtDNA damage and enhanced the binding activity of both NF-κB and AP-1 in OLGs. These results agree with earlier studies showing increases in NF-κB and AP-1 activity in AD brains (Anderson et al., 1994; Kaltschmidt et al., 1997).

NF-κB and AP-1 activation may affect several cellular processes, including cell viability. Reports on NF-κB effects on cell death are contradictory, with NF-κB shown as either cytoprotective (Kaltschmidt et al., 1997; Mattson et al., 2000) or cytotoxic (Grilli et al., 1996). NF-κB and AP-1 are major pro-inflammatory transcription factors. NF-κB and AP-1 activation may cause cell death and tissue destruction via an inflammatory reaction (Barnes and Karin, 1997; Karin et al., 1997). An inflammatory reaction has been noted in AD brains (Griffin et al., 1998; McGeer and McGeer, 1999). Collectively, these findings indicate that activation of NF-κB and AP-1 may have complex effects in Aβ-induced neurodegenerative processes. It is not impossible that Aβ-induced OLG death shown in the present study was mediated by cytokines released by Aβ-activated microglia or astrocytes. The observation that Aβ caused OLG death in differentiated OLGs derived from embryonic preprogenitors of >98% purity in the absence of cells exhibiting astrocyte or microglia markers makes it unlikely that Aβ killed OLGs indirectly by cytokines released from contaminating astrocytes or microglia.

In line with the oxidative hypothesis, we also found that NAC, an antioxidant, was effective in reducing Aβ-induced OLG death. Increasing evidence demonstrates that oxidative stress causes cell dysfunction in age-related disorders such as atherosclerosis and neurodegenerative disorders, including AD (Beckman and Ames, 1998; Behl, 1999; Markesbery, 1999). The brain is especially susceptible to injury via oxidative processes because of its high glucose-driven metabolic rate, low levels of antioxidants, and high concentrations of polyunsaturated fatty acids that serve as substrates for lipid peroxidation (Behl, 1997, 1999; Markesbery, 1999). Aβ enhancement of oxidative stress can be facilitated further by its direct interaction with cell membranes and by induction of oxygen-free radicals including nitric oxide through microglial and astrocyte activation (Behl, 1997; Akama et al., 1998). On the basis of the oxidative hypothesis, antioxidants such as α-tocopherol and selegiline have been used and may delay the progression of AD in patients (Mayeux and Sano, 1999).

In summary, we demonstrate that Aβ is cytotoxic to OLGs. This mechanism of Aβ-induced OLG death remains to be fully elucidated but appears to involve Aβ-induced oxidative stress. The direct cytotoxic effects of Aβ on OLGs raises the possibility that Aβ deposition may affect myelin integrity and thus contribute to white matter dysfunction in AD (Scheltens et al., 1992; Tanaka et al., 1998; Salat et al., 1999). Equally important is the increasing awareness of enhanced brain APP accumulation in the white matter in neurological disorders, including traumatic brain injury (Blumbergs et al., 1994), stroke (Jendroska et al., 1995; Yam et al., 1998), and multiple sclerosis (Ferguson et al., 1997). Increase in Aβ deposition has also been shown after head trauma (Roberts et al., 1991; Smith et al., 1998) and cerebral ischemia (Yokota et al., 1996).

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