Bax Protein Expression Is Increased in Alzheimer's Brain: Correlations with DNA Damage, Bcl-2 Expression, and Brain Pathology

JOSEPH H. SU, MD, GANGMIN DENG, PHD, AND CARL W. COTMAN, PHD

Abstract. We have shown that many neurons in Alzheimer's disease (AD) exhibit terminal deoxynucleotidyl transferase (TdT) labeling for DNA strand breaks, and upregulation of Bcl-2 is associated with neurons exhibiting nuclear DNA fragmentation, while downregulation of Bcl-2 is associated with tangle-bearing neurons in AD brains. Consequently, we examined the expression of bcl-associated X (Bax) protein in AD brain. Immunoreactivity for Bax was seen in neurons and microglia of the hippocampal formation, and was elevated in the majority of AD cases as compared to control cases. Interestingly, 2 transitional cases, which had mild degeneration changes, exhibited relatively high levels of Bax immunoreactivity. Most Bax-positive neurons showed either TdT-labeled nuclei or Bcl-2 immunoreactivity. Although Bax immunoreactivity was detected within most early tangle-bearing neurons, many Bax-positive neurons did not colocalize with later-stage tangle-bearing neurons. In regions containing relatively few tangles in mild AD brains, many TdT-labeled neurons were immunolabeled with Bax antibody and most of them lacked evidence of neurofibrillary changes. These findings suggest that Bax may contribute to neuronal cell death in AD. Furthermore, DNA damage and the upregulation of Bax appear to precede tangle formation or may represent an alternative pathway of cell death in AD.

Key Words: Alzheimer's disease; Bax; Bcl-2; Cell death; DNA fragmentation; Neuropathology.

INTRODUCTION

Neuronal cell death is a prominent feature of Alzheimer's disease (AD) (1–3). Cell death occurs by one of two general pathways, apoptosis or necrosis. Since DNA degradation occurs in both apoptosis and necrosis, both apoptotic and necrotic strand breaks can be detected with new histological techniques, such as TdT-mediated dUTP-biotin nick-end labeling (4), in situ nick translation (5) and in situ end-labeling (6). We have shown that cells in the AD brain exhibit labeling for DNA strand breaks using TdT, and a subset of these cells exhibit the classical, distinct morphological characteristics of apoptosis (condensation of fragmented DNA, nuclear shrinkage, and formation of apoptotic bodies), whereas few or no such nuclei are detected in control brain (7–8). To understand the mechanisms of neuronal degeneration in AD, it will be necessary to examine regulators of multiple cell death pathways.

During the past several years, evidence has accumulated suggesting that the bcl-2 gene family is a common regulator of multiple apoptotic pathways (for review see [9]). Bcl-2 has been shown to inhibit apoptosis and promote cell survival (10–11). Thus, bcl-2 has properties suggestive of an “anti-apoptosis” gene. Recently, it has been suggested that bcl-2 also inhibits the necrosis of neuronal cells induced by glutathione depletion and by decreasing the net cellular production of reactive oxygen species (12–13). Bcl-2 is expressed in most neurons; with aging Bcl-2 expression declines in most neurons of the CNS but is retained in neurons of the PNS (14). We have recently demonstrated that upregulation of Bcl-2 is associated with neurons exhibiting DNA fragmentation, while downregulation of Bcl-2 is associated with tangle-bearing neurons in the AD brain (15).

Bcl-2–associated X protein (Bax) is a member of the bcl-2 gene family. Bax has extensive amino-acid sequence homology with Bcl-2 protein (16) and is expressed in neurons of the central and peripheral nervous system (17). It is known that overexpression of Bax protein induces apoptotic cell death, and the action of Bax appears to be neutralized when heterodimerized with Bcl-2 and some other members of the Bcl-2 protein family that function as suppressors of cell death (16). It has been proposed that the ratio of Bax to Bcl-2 and other anti-apoptotic Bcl-2 family proteins appears to predetermine the life or death response of a cell to an apoptotic stimulus (16).

In the present study, we sought to determine the expression of Bax protein in AD brain and to evaluate Bax expression in relationship to DNA fragmentation, Bcl-2 expression, and paired helical filament (PHF) formation. The results demonstrate that immunoreactivity for Bax was elevated in a majority of AD cases as compared to control cases. Bax immunoreactivity was detected in neurons and microglia. Most Bax-positive neurons showed TdT-labeled nuclei or Bcl-2 immunoreactivity in AD brain. Furthermore, most Bax-positive neurons did not colocalize with PHF, although Bax immunoreactivity was detected within most early tangle-bearing neurons. These findings suggest that Bax may contribute to neuronal cell death in AD. Furthermore, DNA damage and Bax upregulation appear to precede tangle formation and may represent an alternative pathway of cell death in AD.

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TABLE 1

Control and AD Case Information, and Relative Bax Labeling in the Hippocampal Formation

<table>
<thead>
<tr>
<th>Case #</th>
<th>Diagnosis</th>
<th>Age</th>
<th>PMD</th>
<th>MMSE</th>
<th>Bax labeling</th>
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<tr>
<td>1</td>
<td>C</td>
<td>86</td>
<td>4.5 h</td>
<td>n/a</td>
<td>+</td>
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<tr>
<td>2</td>
<td>C</td>
<td>58</td>
<td>4.0 h</td>
<td>n/a</td>
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</tr>
<tr>
<td>3</td>
<td>C</td>
<td>74</td>
<td>8.0 h</td>
<td>n/a</td>
<td>+</td>
</tr>
<tr>
<td>4</td>
<td>C</td>
<td>83</td>
<td>6.0 h</td>
<td>n/a</td>
<td>++</td>
</tr>
<tr>
<td>5</td>
<td>C</td>
<td>75</td>
<td>3.5 h</td>
<td>n/a</td>
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<td>6</td>
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<td>10</td>
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<td>++</td>
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<td>11</td>
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<td>19/30</td>
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<td>21/30</td>
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<tr>
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<td>2.7 h</td>
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</tr>
<tr>
<td>16</td>
<td>AD</td>
<td>83</td>
<td>2.7 h</td>
<td>9/30</td>
<td>++</td>
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</table>

Abbreviations: MMSE, Minimental State Examination; total score 30 and lower numerator represents more severe dementia; n/a, Not available; PMD, Postmortem delay; C, Control case; T, Transitional case with mild to moderate senile degeneration changes insufficient to meet the CERAD criteria of AD.

MATERIALS AND METHODS

Postmortem tissue was obtained from the Institute for Brain Aging and Dementias Tissue Repository. The hippocampal formation from 8 AD and 5 control cases was examined. In addition, three transitional cases with mild to moderate senile degeneration changes insufficient to meet the Consortium to Establish a Registry for the Diagnosis of Alzheimer’s disease (CERAD) criteria of AD (18) were also used for this study. Fresh brain tissue was fixed in 4% paraformaldehyde in 0.1M Sorensen’s buffer, pH 7.3 for 24 hours (h), or fixed in 10% formalin in 0.1 M Sorensen’s buffer, pH 7.3 for 48 to 72 h, and stored in 0.1 M PBS (0.02% sodium azide) at 4°C. All experiments were performed using free-floating 50-μm sections cut on a Vibratome and collected in PBS, pH 7.4. Alzheimer’s disease tissue was taken from neuropathologically defined cases that met the CERAD requirements (18). Alzheimer’s disease, transitional, and control cases were matched for age and PMD as closely as possible (Table 1). Mean age AD = 79 years, control = 75 years; mean PMD AD = 3.9 h, control = 5.6 h.

TdT 3’-OH DNA Strand Break Labeling

ApopTag peroxidase kits (Oncor, MD) were used to detect digoxigenin-nucleotide residues added by TdT to the 3’-OH termini of DNA strand breaks generated during DNA fragmentation in neurons. ApopTag staining was performed according to protocol as described previously (7, 15). Briefly, tissue sections were incubated in TdT enzyme and digoxigenin-dUTP reaction buffer. Sections were then incubated in anti-digoxigenin-antibody conjugated with peroxidase. The digoxigenin-dUTP-peroxidase complex was visualized by reacting with DAB (Vector Labs, CA) to generate a brown reaction product. Negative controls were performed by substituting distilled water for TdT enzyme in the preparation of the working solution and were negative.

Immunocytochemistry

Tissue was processed as described previously (7, 15). Briefly, tissue sections were treated for 20 minutes (min) with 1% H2O2 to inactivate endogenous peroxidases. Sections were incubated overnight at room temperature in primary antibody, rinsed, and incubated in biotinylated secondary antibody and avidin-biotin complex for 1 h, respectively (Vector Labs, CA). The final color product for single labeling was visualized by diaminobenzidine (DAB) for a brown reaction product or an SG kit (Vector Labs, CA) for a blue-gray reaction product. Immunostaining intensity of Bax was rated as follows: +, weak; ++, moderate; ++++, intense. In double-labeling experiments, bound antibodies were detected using a DAB kit for the first antigen and an SG kit for the second antigen. In triple-labeling experiments, bound antibody was detected using FITC- or CY3-conjugated IgG for the third antigen as described previously (19). Sections incubated in parallel without primary antibody failed to develop specific staining.

Antibodies

Two commercially available polyclonal antibodies were used to recognize human Bax. The PharMingen Bax antibody (13666E) recognizes human Bax. A synthetic peptide corresponding to amino acids 43–61 of human Bax was used as an

TABLE 2

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Antigen</th>
<th>Dilution</th>
<th>Source</th>
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<tr>
<td>13666E</td>
<td>Human Bax (43–61)</td>
<td>1:800–1,500</td>
<td>PharMingen, San Diego, CA</td>
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<tr>
<td>PC66</td>
<td>Human Bax (150–165)</td>
<td>1:30–50</td>
<td>Calbiochem, Cambridge, MA</td>
</tr>
<tr>
<td>sc-509</td>
<td>Human Bcl-2</td>
<td>1:40</td>
<td>Santa Cruz Biotechnology Inc., Santa Cruz, CA</td>
</tr>
<tr>
<td>AT8</td>
<td>Early phosphorylated PHF/tau (Ser-202)</td>
<td>1:5,000–20,000</td>
<td>Innogenetics, Belgium</td>
</tr>
<tr>
<td>PHF-1</td>
<td>PHF/tau (Ser-396)</td>
<td>1:200–1,000</td>
<td>Dr Sharon G. Greenberg, Dementia Research, Burke Medical Res., White Plains, NY</td>
</tr>
<tr>
<td>LN3</td>
<td>HLA-Dr</td>
<td>1:50–100</td>
<td>ICN, CA</td>
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immunogen (20). The specificity of this antibody was verified by immunoprecipitation, Western blot analysis, and immunohistochemistry (20). The Calbiochem Bax antibody (PC66) was generated with a peptide corresponding to residue 150–165 of the human Bax protein. This antibody recognizes human, opossum, and mouse Bax. One monoclonal antibody (sc-509) was used to recognize human Bcl-2. This antibody has been previously characterized by immunoprecipitation and Western blot analysis, where clone 100 detected native Bcl-2 at the correct molecular weight under both reducing and nonreducing conditions. Further, sc-509 was not cross-reactive with either Bax 21 or Bcl-x (Santa Cruz Biotechnology, CA). Appropriate positive and negative tissue controls have also been previously published (15, 21). Two well-characterized monoclonal antibodies, AT8 and PHF-1, were also used to determine whether Bax-positive neurons were present within neurofibrillary tangles (NFTs). AT8 was used as a marker for pretangle and early tangle-bearing neurons, whereas PHF-1 was used as marker for late tangle-bearing neurons (19, 22–24). The specificity of these two antibodies have been described in detail previously (22, 25, 26). An antibody against nonpolymorphic HLA-Dr antigen was used to determine whether Bax immunoreactivity was present within microglia cells.

RESULTS

In general, both Bax antibodies immunostained neurons and microglia. No significant differences in staining were observed between the two Bax antibodies. Furthermore, there was no apparent relationship of Bax immunostaining with age, PMD, or different fixation protocol.

Bax Expression in Age-matched Control and Transitional Cases

Bax localization was examined in 5 cases of nondemented normal controls and 3 transitional cases with an age range similar to the AD cases. Bax immunoreactivity was found to be present in some neurons, primarily in pyramidal and granular cells. Neurons positive for Bax showed weak to moderate staining within the soma and proximal apical dendrites (Fig. 1) in all 5 cases. In 3 transitional cases, relatively high Bax immunoreactivity was found within the soma and dendrites of many neurons. Interestingly, these 3 transitional cases had no clinical history of AD, but upon neuropathological evaluation exhibited mild to moderate senile degeneration insufficient to meet the CERAD criteria (18) of AD (Table 1). The pattern of Bax expression displayed a laminar distribution profile. Neurons in layer III and V of entorhinal cortex, pyramidal cell layer of hippocampus, and granular cell layer of dentate gyrus showed stronger immunoreactivity for Bax compared to other layers.

In all control and transitional cases, light to moderate immunoreactivity for Bax was also found within glial cells with morphology resembling that of microglia (Fig. 1).

Bax Expression in AD Brain

In general, Bax immunoreactivity was more intense in 8 AD brains examined compared to the majority of control cases (Table 1). Staining for Bax did not appear to increase with the severity of dementia (Table 1). Neurons immunopositive for Bax in AD cases were clearly detected in pyramidal cell bodies and neurites (Fig. 2a). Granular cells of the dentate gyrus also showed intense Bax immunoreactivity. Bax immunostaining was located primarily in the soma in a granular, punctate pattern in those cells. Occasionally, Bax immunoreactivity was detected within some neuronal nuclei.

Bax immunoreactivity was also observed within some glial cells. These positive glial cells with increased perikaryal cytoplasm and shorter and thicker processes were located within neuropil or grouped around plaque areas...
(Fig. 2b). The immunoreactivity for Bax-positive glial cells appeared to be stronger than that in the control brains examined. Double-labeling experiments for Bax and HLA-Dr revealed that most Bax-immunopositive glial cells were microglia (not shown).

Co-localization of Bax Immunoreactivity and DNA Damage within Neurons in AD Brain

Bax immunostaining combined with TdT end-labeling was used to evaluate Bax expression in relationship to DNA fragmentation. All of the AD patients examined in this study exhibited a prominent co-localization between neurons exhibiting immunoreactivity for Bax- and TdT-positive nuclei (Fig. 3). Most of the cells double labeled for TdT and Bax were pyramidal neurons and were distributed throughout the entorhinal cortex and CA1. A subset of these double-labeled cells exhibited the classical, distinct morphological characteristics of apoptosis (condensation of fragmented DNA, nuclear shrinkage, and formation of apoptotic bodies) (Fig. 3, inset). Although many neurons with strong Bax staining were associated with intensely TdT-labeled nuclei, a subset of neurons with intensely TdT-labeled nuclei did not associate with strong or moderate Bax staining (Fig. 3). Furthermore, less than 10% of the neurons exhibiting strong immunoreactivity for Bax did not exhibit TdT-positive nuclei.

Relationship of Bax-positive Neurons to Bcl-2 Immunoreactivity in AD Brain and Transitional Cases

In agreement with our previous findings, Bcl-2 was upregulated in AD brains (15, 21). Interestingly, Bcl-2 was also upregulated in three transitional cases. Staining for Bax and Bcl-2 was conducted using DAB/SG double labeling to evaluate the relationship of Bax-positive neurons to Bcl-2 immunoreactivity. Examination of neurons double-labeled for Bax and Bcl-2 revealed that the majority of Bax-positive neurons co-localized with Bcl-2 (Fig. 4a).

While Bcl-2 immunoreactivity was detected within the majority of Bax-positive neurons, a few Bcl-2 positive neurons did not co-localize with Bax (Fig. 4b) in both AD and transitional cases, suggesting that Bax was less widely expressed than Bcl-2 in the hippocampal formation. Such single-labeled neurons were easily found within the CA1 region, entorhinal cortex, and granular cells of the dentate gyrus.

Relationship of Tangle-bearing Neurons to Bax Immunoreactivity and DNA Damage in AD and Transitional Cases

Staining for Bax, AT8, or PHF-1, and TdT end-labeling was conducted using DAB/immunofluorescence and DAB/SG double-labeling or DAB/SG/ immunofluorescence triple-labeling to confirm that these observations were not due to quenching. In AD cases, examination of neurons double-labeled for Bax and PHF-1 revealed that most PHF-1-positive neurons exhibited negative or weak immunoreactivity for Bax (Fig. 5). As described previously, PHF-1 immunopositive deposits usually appeared either as fibrillar inclusions filling almost the entire soma or as large bundles of loosely arranged filament, suggesting that these cells were later-stage tangle-bearing neurons (19, 24).

In contrast, examination of neurons double-labeled for Bax and AT8 revealed that most AT8-positive neurons co-localized with strong Bax immunoreactivity (Fig. 6). In agreement with our previous observations and others (23, 24), AT8-positive deposits appeared as granular inclusions or fibrillar inclusions within part of the soma, suggesting that they were pretangle or early tangle-bearing neurons. It is interesting to note that in three transitional cases with relatively high Bax immunoreactivity, a few isolated pretangle or early tangle-bearing neurons with strong Bax immunoreactivity were dispersed among numerous strong Bax-positive neurons that lacked evidence of PHF suggesting that upregulation of Bax may occur prior to tangle formation (Fig. 7). Furthermore, in mild AD cases which exhibited relatively few tangles in some regions, most Bax-positive neurons with TdT-positive nuclei did not co-localize with either AT8 or PHF-1 (Fig. 8), suggesting that tangle formation may occur after both Bax upregulation and DNA fragmentation or can operate as an independent pathway in some neurons.

DISCUSSION

In this report, we have demonstrated that Bax protein is upregulated in AD in comparison to control cases. Furthermore, we have shown that there is a prominent co-localization between neurons exhibiting immunoreactivity for Bax and TdT-positive nuclei. We also demonstrated that Bax immunoreactivity is upregulated within pretangle and early tangle-bearing neurons as labeled by AT8 antibody. These results are consistent with recent studies both in vitro and in vivo, suggesting that Bax is activated during cell degeneration and overexpression of Bax accelerates cell death (16, 27, 28). Our data suggest the hypothesis that Bax may have an important role in the regulation of neuronal cell death in AD. If this hypothesis is true, there are a few possible explanations for the lack of colocalization between Bax-positive and TdT-positive cells. First, the lack of complete colocalization between Bax-positive and TdT-positive cells may suggest that other proteins participate in neuronal cell death in AD. This is consistent with the data reported in the present study showing that a significant subset of Bcl-2 positive neurons did not co-localize with Bax. It should be noted that Bcl-2 and Bax are not the only known members of the Bcl-2 protein family expressed in brain. Furthermore, other members of the Bcl-2 family, including
Bad and Bak, have been shown to enhance cell death and antagonize the protective effects of Bcl-2 in a dominant fashion (16, 29–31), though it remains unknown at present whether these proteins are expressed in neurons. Second, upregulation of Bax protein level may not in itself be sufficient for cell death, since Bcl-2 or other proteins counteract Bax. It may also be speculated that TdT-positive nuclei may occur prior to Bax upregulation. Such TdT labeling may reflect DNA damage rather than the process of cell death and could represent an active process of DNA damage/DNA repair in cells at risk or an accumulation of cells beginning to undergo apoptosis (8, 15), Bax not yet induced. Third, though Bax protein levels are elevated in AD brains, increases in Bax are probably not required for all types of neuronal death. This is consistent with the recent report suggesting that glutamate-induced death of cerebellar granular cells in culture does not require increased Bax expression in some types of neurons (32). Thus, it is likely that upregulation of cell death promoting protein Bax may not be associated with all cell death in AD.

Neurofibrillary tangles are one of the major pathological features of AD and are hypothesized to be a fundamental event leading to neuronal cell death. The mechanism(s) and molecular process related to this event have not been identified. It has been known that NFTs exhibit a well-defined pattern of formation, permitting the differentiation of stages (33). In early stages, only mild NFT involvement appears in the hippocampus (CA1) and deep layers of entorhinal region. In this study, we found that overexpression of Bax protein was associated with both pretangle neurons and early tangle-bearing neurons (AT8-positive), suggesting that Bax may participate in cell death of some tangle-bearing neurons. However, it is particularly worth noting that in three transitional cases with mild degeneration and relatively high levels of Bax immunoreactivity, a few isolated NFTs are dispersed among many neurons with moderate to strong Bax staining, without evidence of PHF formation. These results are consistent with our observations regarding the sequence of tangle formation and cells double-labeled for TdT and Bcl-2. These data may reflect a sequence of events in which DNA is initially damaged, followed by Bax and Bcl-2 upregulation, then tangle formation and cell death. Alternatively, these observations could reflect two different pathways of DNA damage in the AD brain, one that involves tangle formation and one that does not (15), although these are not mutually exclusive processes.

Bax protein is expressed in neurons but not in glial cells of the normal young mouse central nervous system (17). Thus, an unexpected finding of the present study was that Bax immunoreactivity was present and upregulated in microglia of AD brain. This result is unlikely to be due to nonspecific artifacts, because the specificity of Bax antibody (13666E) was verified by immunoprecipitation, Western blot analysis, and immunocytochemistry, as well as by comparison with preimmune serum and competition experiments (20). Furthermore, similar results also were observed with anti-Bax monoclonal antibody. Thus, the differences may be attributed to age or, alternatively, may be species-specific.

Microglia has been suggested to have a central role in enhancing the development of AD towards β44 deposition, neuronal isolation, and system disconnection (for review see [34]). Thus, the increased immunoreactivity for Bax in microglia may reflect adverse pathophysiological processes in the AD brain. For example, cultured microglia are vulnerable to β-amyloid toxicity in vitro (35).

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**Fig. 3.** Co-localization of TdT labeling (brown) and Bax immunoreactivity (blue-gray) in AD, ×180. Note that there is a prominent co-localization between neurons exhibiting immunoreactivity for Bax- and TdT-positive nuclei (arrows). Arrowheads indicate neurons with intensely TdT-labeled nuclei do not associate with Bax staining. Inset: Higher magnification showing the classical, distinct morphological characteristics of apoptosis (chromatin aggregation) (×750).

**Fig. 4.** Co-localization of Bax (brown) and Bcl-2 (blue-gray) immunoreactivity in transitional cases, ×180. (A) The majority of Bax-positive neurons co-localize with Bcl-2 (arrows). (B) A significant subset of Bcl-2-positive neurons do not co-localize with Bax (asterisks).

**Fig. 5.** Relationship of PHF-1-positive neurons to Bax immunoreactivity in AD, ×130. (A) Bax-positive neurons (arrows). (B) Same section showing weak or no immunoreactivity for PHF-1 in Bax-positive neurons (arrows). In contrast, PHF-1-positive neurons are not usually co-localized with Bax immunoreactivity (asterisks).

**Fig. 6.** Co-localization of Bax (brown) and AT8 (blue-gray) immunoreactivity in transitional cases, ×130. The majority of Bax-positive neurons co-localize with AT8 immunoreactivity (arrows).

**Fig. 7.** Most strong Bax-positive neurons (asterisks) lack evidence of PHF in transitional cases, ×130. Note that a few isolated pretangle or early tangle-bearing neurons with strong Bax immunoreactivity are dispersed among numerous Bax-positive neurons (arrows).

**Fig. 8.** Relationship of TdT-positive nuclei (brown) with or without immunoreactivity for Bax (blue-gray) to tangle-bearing neurons (red) labeled with AT8/PHF-1 antibodies in CA1 of mild AD, ×130. (A) Numerous Bax-positive neurons with TdT-positive nuclei (asterisks). (B) Same section showing weak or no immunoreactivity for AT8/PHF-1 in Bax-positive neurons with TdT-positive nuclei (asterisks). Arrows indicate AT8/PHF-1-positive neuron.
Alternatively, it may also reflect microglia autodestruction, which may have a prominent role in AD (36). Further investigations are required to evaluate the significance of Bax overexpression in microglia.

In conclusion, our findings suggest that Bax protein is present in both neurons and microglia, and is upregulated in AD relative to control cases. Overexpression of Bax protein and TdT-labeling, PHF, or Bel-2 exhibit a high, but not complete, degree of colocalization in AD brain. These data suggest the hypothesis that Bax protein may have an important role in regulating early events associated with neuronal cell death in AD.

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