Extracellular deposition of beta-amyloid upon p53-dependent neuronal cell death in transgenic mice.

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Research Article

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Extracellular Deposition of β-Amyloid upon p53-dependent Neuronal Cell Death in Transgenic Mice

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Abstract

The finding that intracellular expression of the β-amyloid protein (Aβ) under a neuron-specific promoter led progressively to degeneration and death of neurons in the brains of transgenic mice provides a unique opportunity to utilize this animal model to both understand the mechanism that underlies neuronal cell death and define the complexity of events which may ensue. We observed a correlation between Aβ accumulation in selective neurons and activation of p53, a protein that has been implicated in the induction of apoptosis. Histological and immunohistochemical evaluations of adjacent brain sections suggest that expression of p53 is accompanied by nuclear DNA fragmentation. In certain regions with marked neuronal cell death, extracellular deposition of Aβ became evident, together with the local activation of astrocytes. Interestingly, the neuritic structures underlying the Aβ deposits showed altered synaptophysin immunoreactivity and morphologic evidence for damage. This transgenic mouse model suggests that intracellular generation of the Aβ protein not only leads to the death of the neuron but may also functionally impair neighboring neurons as well. It further offers a mechanism whereby neuritic plaques may be derived. (*J. Clin. Invest.* 1996. 98:1626–1632.) Key words: Alzheimer's disease • Aβ peptide • neuritic plaques • apoptosis • p53

Introduction

A fundamental question in understanding the role of the β-amyloid protein (Aβ)[1] in Alzheimer's disease (AD) is to determine the mechanism by which this 42-amino acid peptide induces neuronal cell death. While there is increasing evidence for Aβ being derived from the β-amyloid precursor protein (APP) inside the cell (1–6), it remains obscure as to how the peptide gets to be deposited on the outside in the extracellular spaces. It is believed that, subsequent to secretion, diffuse Aβ deposits can form fibrillar aggregates which, only then, become cytotoxic to neurons in the vicinity (7). Since the processing of APP to Aβ occurs in normal individuals as well, is presumed the development of AD to proceed as a consequence of changes in either the quantity or quality of Aβ (1–3). Indeed, missense mutations in the APP gene either around or within the Aβ coding region have been identified in some familial cases of AD which may facilitate Aβ production or alter its properties (5, 8, 9).

Much of this proposed sequence of events has been suggested by histopathological observations made with brain tissues from AD and Down syndrome patients and by biochemical studies using cells in culture (10). Valuable as they have been, neither approach would precisely define either the progressive morphological changes or the functional consequences of Aβ expression. As an alternative strategy, we have derived transgenic mice with intracellular expression of Aβ specifically in neurons, with the hope of defining the successive steps which may reflect AD pathogenesis (11). Since these mice develop extensive neuronal cell degeneration and death, characteristics which may be considered the ultimate endpoints of AD, they provide an opportunity to define how intracellular Aβ causes cell death and whether such dying neurons are the source of the extracellular Aβ.

Methods

Histological analysis. Brain sections from perfused animals were processed as described previously. Sections of paraffin embedded tissues were cut at 5-μm thickness and stained with hematoxylin and eosin, Bielshowsky's silver stain, thioflavin, and periodic acid Schiff for histological examination (11). Transgenic brains were analyzed in parallel with nontransgenic brains.

Immunohistochemistry. For immunohistochemical staining of p53, brain sections of paraffin embedded brain samples were mounted on silane-coated slides, dewaxed, rehydrated, and treated with 0.1 mg/ml trypsin for 20 min at room temperature. Sections were blocked with normal goat serum and incubated overnight at 4°C with an affinity-purified rabbit polyclonal antibody to p53, designated anti-p53N (12). Immunohistochemical staining for Aβ has been previously described (11). All immunostained sections were developed with diaminobenzidine substrate using the avidin-biotin horseradish peroxidase system (Vector Laboratories, Burlingame, CA).

TUNEL (terminal deoxynucleotide transferase-mediated dUTP-biotin nick-end labeling) analysis. Paraffin sections were dewaxed, rehydrated, and digested with proteinase K (20 μg/ml). Endogenous peroxidase activity was quenched by treatment in 3% hydrogen peroxide. Sections were then rinsed in water and labeled at 37°C for 30 min with terminal deoxynucleotide transferase (0.5 U/μl) in a cocktail consisting of 25 mM Tris pH 6.6, 200 mM potassium cacodylate, 1 mM $CoCl_2$, 0.25 mg/ml bovine serum albumin, and 2 μM biotin-dUTP. Sections were rinsed and incubated with avidin and biotinylated horseradish peroxidase (Vector Laboratories), rinsed in water and then stained with diaminobenzidine.

Electron microscopy. Transgenic and nontransgenic mice were transcardially perfused with 4% paraformaldehyde/2% glutaraldehyde in PBS. The hippocampus, cerebral cortex, and thalamus were

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1. *Abbreviations used in this paper:* AD, Alzheimer's disease; AP, β-amyloid protein; APP, β–amyloid precursor protein; H&E, hematoxylin and eosin; TUNEL, terminal deoxynucleotide transferase-mediated dUTP-biotin-nick-end labeling.

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dissected and cut into 1-mm pieces and further incubated in the fixative solution described above. After 3 h, the tissue pieces were transferred to PBS, and embedded and processed for transmission electron microscopic analysis.

Results

The transgenic mice were generated by using a 1.8-kb DNA fragment derived from the 5′ flanking region of the mouse neurofilament-light gene to drive expression of the mouse 42-amino acid Aβ protein (11). Expression of the transgene was confirmed by [1] Northern blot hybridization analysis which revealed the predicted 500–600 nt transcript in the brain, [2] in situ hybridization analysis using a transgene-specific probe which showed neuronal accumulation of the Aβ mRNA throughout the brain, and [3] immunohistochemical staining with Aβ specific antibodies which demonstrated cytoplasmic localization of the Aβ protein in only selective regions of the brain (11).

To determine whether the anti-Aβ reactive material display the tinctural properties of insoluble β-amyloid, fixed sections of brain tissue were stained with thioflavin S, a fluorescent amyloid specific dye (13). Thioflavin S-reactive deposits were found exclusively in the cytoplasm of some of the neurons, and the punctate staining pattern suggested their accumulation within vesicles (Fig. 1, *a* and *b*). No fluorescence was detected in the brains of control mice similarly stained with thioflavin S. This finding suggests that thioflavin S deposits were coincident with a subset of anti-Aβ reactive neurons. Additionally, while the anti-Aβ antibody could also detect APP, the detection of thioflavin S fluorescence selectively in the brains of the transgenic mice is consistent with the Aβ immunoreactivity being Aβ and not APP.

We also demonstrated that neurons from the transgenic mice revealed extensive intranuclear DNA fragmentation, detected in tissue sections using the TUNEL method (14), thus providing evidence for cell death in the brain. While the TUNEL procedure is useful for identification of dying cells in situ, there is concern that it may not distinguish between apoptosis and necrosis. To better evaluate the pathway by which the neurons die in the transgenic brains, transmission electron microsopic analysis was performed. Cells dying by apoptosis are characterized by having chromatin that is pyknotic and

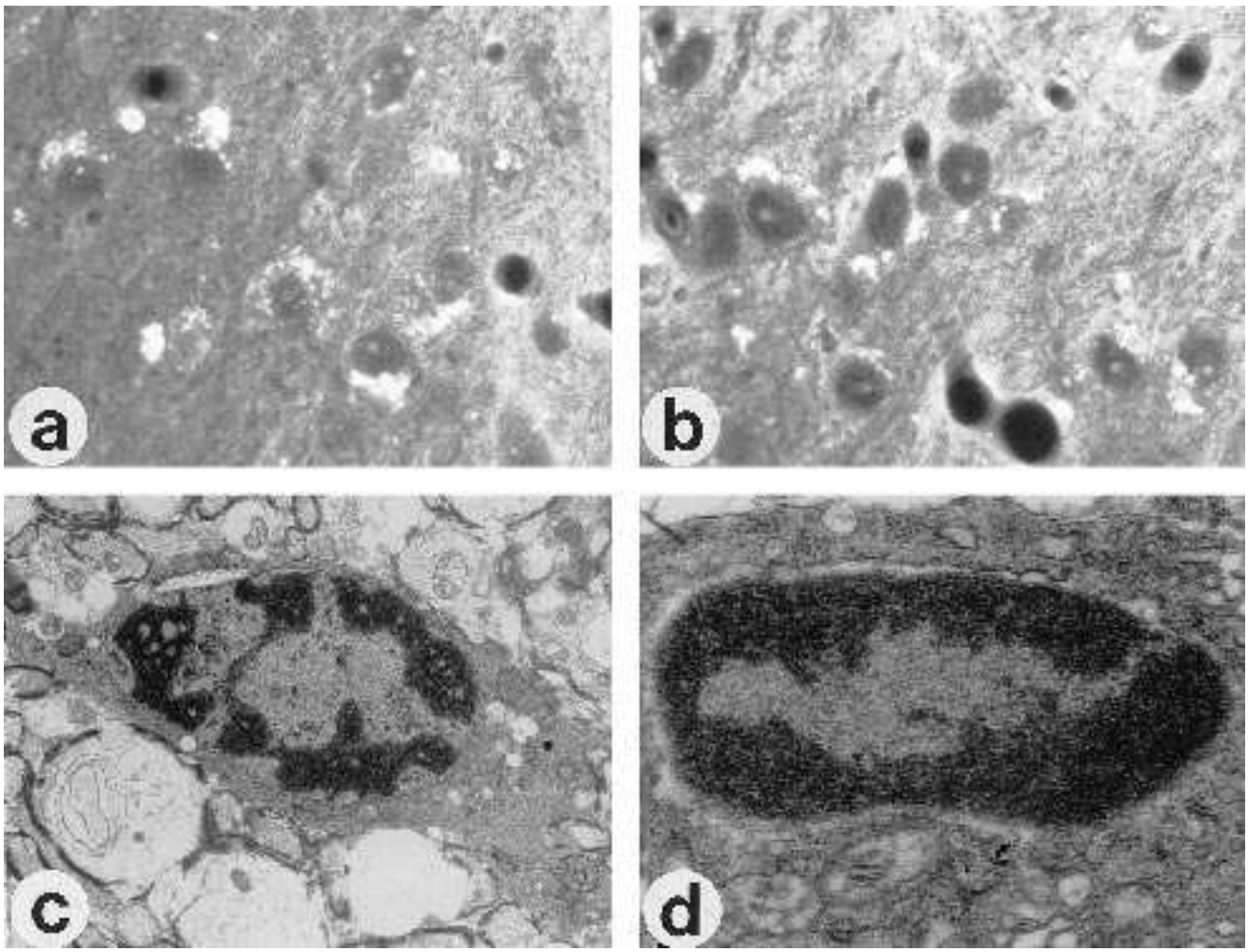

Figure 1. Characterization of neurons in the Aβ transgenic mice. (*a* and *b*) Thioflavin S staining of brain sections from transgenic mice with Aβ-immunoreactive neurons. Thioflavin S-reactive deposits were detected selectively in the cytoplasmic compartment of the cells. (*c and d*) Electron microscopic analysis of brain tissues from transgenic mice with TUNEL positive neurons. Characteristic margination of chromatin against the nuclear membrane was seen in neurons from transgenic mice but not from control littermates.

packed into smooth masses applied against the nuclear membrane (15). As shown in Fig. 1, *c* and *d*, cells with condensed chromatin material were present in the transgenic brains but not found in control littermates. Therefore, morphological as well as biochemical criteria are consistent with neurons in the transgenic mice dying by apoptosis.

Is p53 involved in Aβ-induced neuronal cell death? Many of the Aβ transgenic mice succumbed prematurely and, at about the time of death, all showed multifocal areas of apoptotic cells, particularly in the cerebral cortex, hippocampus, thalamus, and amygdala. The molecular mechanisms underlying apoptosis in different cell types are rapidly being unraveled (16). Specific genes responsible for inducing apoptosis have been found to be activated in certain cell types while those responsible for inhibiting the same outcome appear to be repressed in other cell types (17). Of particular interest is p53, a protein with pleiotropic functions depending on the state of the cell; it can variously induce programmed cell death, suppress normal cell growth, and facilitate DNA repair (18). Since adult neurons are predominantly postmitotic and rarely exposed to genotoxic agents, a basal level of p53 expression is not required. While apoptosis can proceed by either p53 dependent or independent pathways even in the same cell type (19, 20), it is attractive to consider the involvement of p53 in this case since its activation in neurons has been observed in response to ischemia and excitotoxicity (21, 22).

Using an antibody directed against the N terminus of p53 (12), we confirmed by immunostaining of formalin-fixed brain sections that neurons in the cerebral cortex of control mice do not normally express p53 (Fig. 2 *a*). In contrast, cortical neu-

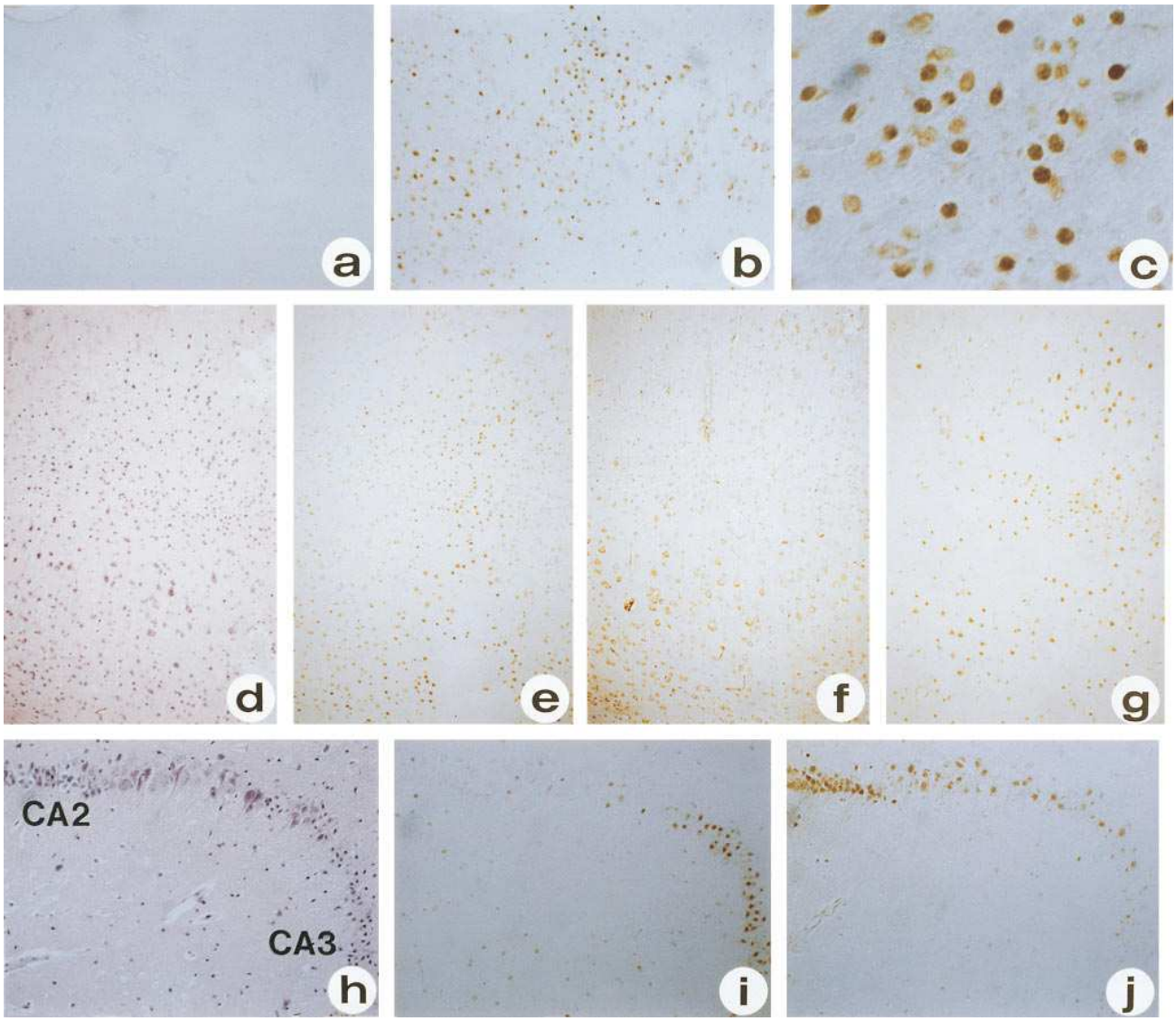

Figure 2. Expression of p53 in Aβ-induced neuronal cell death. Anti-p53 immunoreactivity is not detected in the cerebral cortex of a control mouse (*a*) but is present in abundance in a comparable region of a 10-mo-old Aβ transgenic mouse (*b*). A high magnification view reveals the nuclear localization of p53 in a majority of immunoreactive cells (*c*). Correlation between activation of p53 and Aβ-induced apoptosis is achieved by analysis of serial sections of the neocortex from a 12-mo-old Aβ transgenic mouse, stained with either H&E (*d*), TUNEL (*e*), anti-Aβ (*f*), or anti-p53 (*g*). Evidence for p53 expression inducing apoptosis in the hippocampus of a 10-mo-old transgenic mouse is suggested by analysis of serial sagittal sections stained with either H&E (*h*), TUNEL (*i*), or anti-p53 (*j*). The stained sections were photographed at either ×25 (*d–g*), ×50 (*a*, *b*, *h–j*), or ×150 (*c*).

rons from Aβ transgenic mice ($n = 10$) showed extensive p53 immunostaining (Fig. 2 *b*). However, not every neuron was found to express p53; the extent of involvement varied from subregion to subregion and from mouse to mouse, perhaps suggesting that the intrinsic ability to express intracellular Aβ was not sufficient. The addition of the synthetic peptide to which the antibody was derived completely blocked p53 immunostaining (data not shown). It is interesting to note that while a majority of the detectable cortical neurons in this transgenic mouse showed predominantly nuclear p53 immunostaining (Fig. 2 *c*), a significant subset showed both nuclear and cytoplasmic localization (Fig. 2 *b*, *bottom left*), and an occasional subset frequently arranged as a cluster may exhibit only cytoplasmic accumulation (Fig. 2 *b*, *right*). It is not clear whether this difference in subcellular localization of p53 represents different functional states of the cells or whether it characterizes responses of different neuronal subtypes. Regardless, activation of p53 is a distinguishing feature of the Aβ transgenic mice and correlates well with recent in vitro studies which suggest that Aβ induces oxidative injury (23–25), an established inducer of p53 (26).

Does activation of p53 correlate with Aβ accumulation? To determine whether sustained expression of Aβ is required to induce p53, we selected for analysis an Aβ transgenic mouse with a well circumscribed area of apoptotic cells juxtaposed to an apparently normal area. As dying cells are characterized by the presence of excessive DNA 3′-hydroxyl ends, they can be detected in tissue sections using the TUNEL method (14). From the relative signal strengths of the labeled nuclei, it may even be possible to distinguish cells in early stages of the apoptotic pathway with marginal morphological changes from cells in advanced stages that have condensed nuclei.

Serial sections of the neocortex from the Aβ transgenic mouse were subjected to hematoxylin and eosin (H&E), TUNEL, anti-Aβ and anti-p53 staining (Fig. 2, *d–g*, respectively). Histological evaluation revealed that the cortical neurons on the top-left side of Fig. 2 *d* were morphologically unaltered and exhibited an open (normal) nuclear morphology. These cells were TUNEL negative and showed no evidence of Aβ accumulation or p53 activation. In contrast, the cortical neurons located along the bottom portion of Fig. 2 *d* revealed a distinctively basophilic cytoplasm and appeared degenerative. These cells were weakly TUNEL positive and showed evidence of both cytoplasmic Aβ and nuclear p53 immunoreactivity. The cortical neurons at the top right portion of Fig. 2 *d* showed highly condensed or fragmented nuclei and were strongly TUNEL positive; many of these cells also expressed Aβ and p53. Since p53 immunoreactivity is not detectable in normal, nontransgenic brains, it suggests that transgene expression (and, in particular, intracellular Aβ accumulation) can be correlated with activation of p53.

Since experimental conditions for exposure of the Aβ and p53 epitopes recognized by the respective antibodies in fixed paraffin sections are quite different, we have not been able to directly demonstrate the two functional markers co existing in the same cell. However, since a majority of neurons in the involved subregion of the brain showed Aβ or p53 immunostaining in successive tissue sections, we believe that we are correct in concluding that both molecules are found in the same cells.

Does p53 activation precede cell death? Since analysis of the cerebral cortex hinted to the expression of p53 occurring before the detection of overt cell death, we analyzed another region of the brain to determine whether even more convincing evidence may be obtained. Serial sections of the hippocampus from another Aβ transgenic mouse were again subjected to H&E, TUNEL and anti-p53 staining (Figs. 2, *h–j*, respectively). The hippocampal neurons were observed to undergo progressive morphological changes along the horn of Ammon. In the CA2 subregion, the cells appeared fairly normal, showed no TUNEL labeling but already had abundance of nuclear p53 immunoreactivity. In contrast to control mice, one can conclude that p53 had been activated although the cells had not undergone apoptosis based on TUNEL analysis. At the junction between the CA2 and CA3 subregions, the cells had acquired significant morphological changes and displayed a markedly altered nuclear to cytoplasmic ratio. These cells became weakly TUNEL positive but maintained a high level of nuclear p53 immunoreactivity, suggesting that p53 is required for these cells to enter the apoptotic pathway. Toward the CA3 subregion, the cells had condensed chromatin structures and nuclei, and were highly TUNEL positive but were no longer p53 immunoreactive. We interpret this finding to be consistent with the activation of p53 before the onset of apoptosis and its disappearance late in the process, although it cannot be ruled out that p53 expression may be induced in cells surviving the stress of Aβ toxicity.

That different brain regions from different animals showed involvement is noteworthy. Despite the variability in the age of onset of neurodegenerative changes, a constant level of transgene mRNA expression was detected by Northern blot hybridization analysis of brain tissues from mice ranging in age from 4 wk to 18 mo (data not shown). This may suggest that the genetic ability to express Aβ is in itself insufficient to assure histopathological changes but that epigenetic or environmental cofactors may play an important role in the disease process.

Is there extracellular deposition of Aβ in the brain? Although greater than 50% of the transgenic mice died by 12 mo-of-age, there were some animals that survived and reached 18–24 mo (11). Using an anti-Aβ antiserum (4G8) that is well characterized and widely used (27), as well as with antisera that we ourselves have generated, we have detected extracellular Aβ immunostaining in some of the transgenic mice analyzed. The 4G8 antibody recognizes an epitope, consisting of amino acids 17–24 of Aβ, which is perfectly homologous between the mouse and human peptides. Although we cannot exclude the possibility that 4G8 recognizes APP as well, it is relevant to note the lack of immunoreactivity in nontransgenic brain sections. As predicted, these deposits were found in different transgenic brain regions, particularly within the neocortex, hippocampus, and thalamus.

Fig. 3 *a* shows an anti-Aβ stained section from a 20-mo-old transgenic mouse, with three distinct regions of extracellular Aβ deposition. The locations of the three general areas can be deduced from parallel brain sections treated with either H&E (Fig. 3 *b*) or Bielschowsky's silver stain (Fig. 3 *c*). The top left region includes the CA1 subregion of the hippocampus and the overlying white matter (Splenium corporis callosi). The top right region lies within the CA3 subregion of the hippocampus and the overlying white matter (Truncus corporis callosi). The bottom left region is located within the thalamus (possibly Nucleus ventralis thalami, pars basalis). A high magnification view of the anti-Aβ immunostaining in all three regions shows that the extracellular deposits resemble diffuse

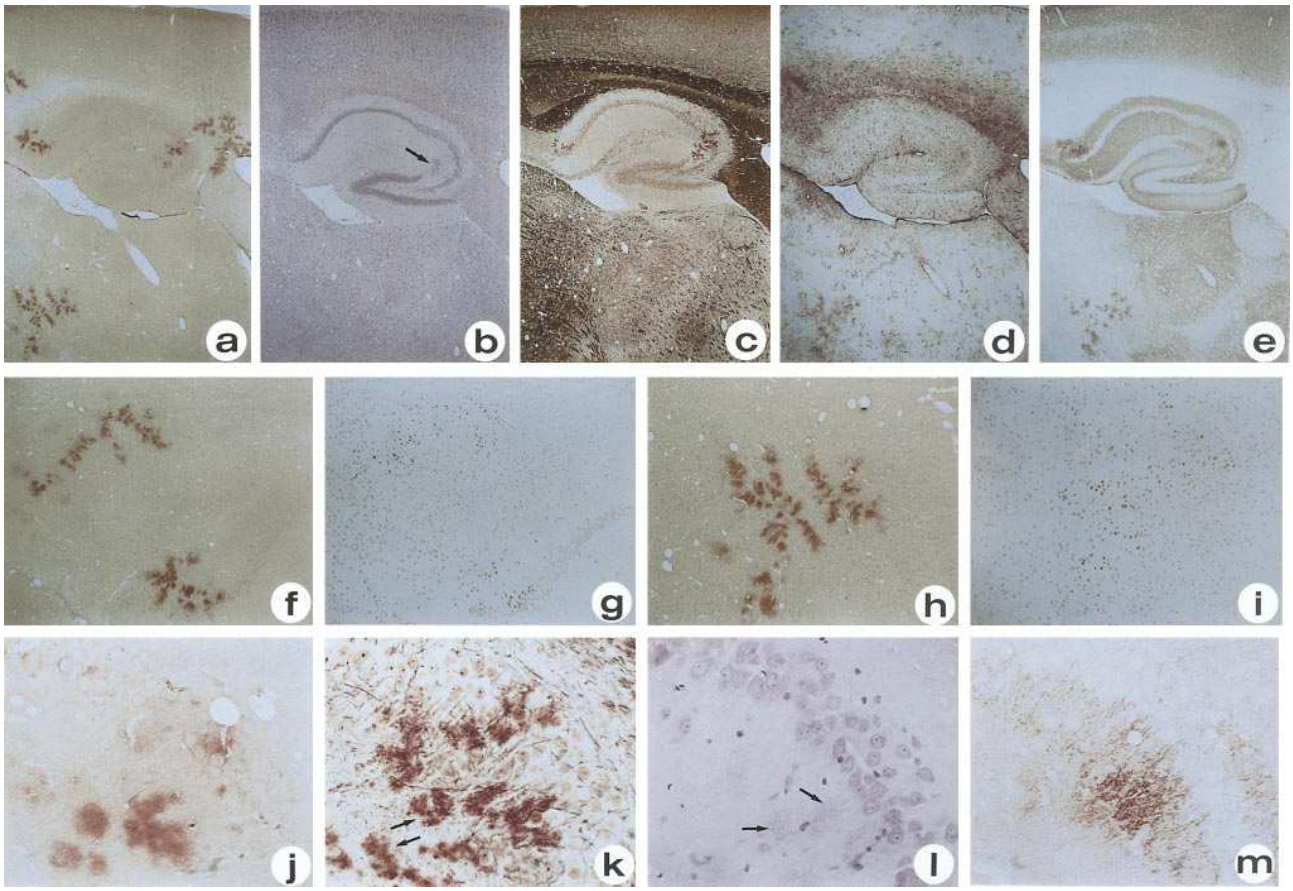

Figure 3. Extracellular deposition of Aβ upon neuronal cell death. Serial sagittal sections of the brain from a 20-mo-old Aβ transgenic mouse were stained with either anti-Aβ (*a*, *f*, *h*, and *j*,), H&E (*b* and *l*), Bielschowsky's silver stain (*c* and *k*), anti-GFAP (*d*), anti-synaptophysin (*e* and *m*), or TUNEL (*g* and *i*). The stained sections were photographed at either ×10 (*a–e*), ×25 (*f–i*) or ×150 (*i–m*). Each antibody yielded a distinct pattern of staining. The low and high magnification views of the Aβ immunoreactivity were not all from the same anti-Aβ stained section, and illustrate the reproducibility of Aβ detection in serial sections.

plaques (Fig. 3, *f*, *h*, and *j*). Interestingly, these deposits could also be detected with facility using a silver stain when found not overlying nerve fibers (Fig. 3 *c*). Not only does this imply significant amounts of the Aβ protein being deposited in the extracellular space, a high magnification view also showed that these deposits assume an insoluble appearance and may represent fibrillar aggregates (Fig. 3 *k*).

Could the extracellular deposits come from dying cells? Recognizing that a dying neuron has an abundance of cytoplasmic Aβ, it is important to determine whether Aβ could be released into the immediate extracellular space upon the death of the cell. This is particularly relevant since the manner by which Aβ gets out of the cell in AD has not been defined. Analysis of the hippocampal CA1 and CA3 cells, in the vicinity of the Aβ deposits, showed evidence of significant cell degeneration. For example, based upon comparable histological evaluation of nontransgenic brain tissue, there is a recognizable paucity of neurons within the CA3 subregion and some of the remaining cells have nuclei that were poorly stained by hematoxylin (Fig. 3 *l*) and nucleoli that could not be identified by Bielschowsky's silver stain (Fig. 3 *k*).

More significantly, TUNEL positive cells were found to perfectly overlap regions with extracellular Aβ immunostaining (Fig. 3, *g* and *i*), confirming the association of cell death with extracellular deposition. Further evidence for cellular injury was obtained from H&E staining of regions around CA1 and CA3, which appeared somewhat hypocellular. The presence of focal regions of basophilia, detected by hematoxylin which stains nuclear chromatin and overlying Aβ immunoreactive areas, suggested that these deposits were released subsequent to cell death (Fig. 3 *b*, *arrows*). At high magnification, an occasional cell ghost was detected together with disorganization of the surrounding neuropil in an area marked by distinct basophilia (Fig. 3 *l*, *arrow*).

Does extracellular Aβ deposition have functional consequences? Due to the localized nature of the Aβ deposits and its insoluble appearance, it is possible that the underlying neurons as mere bystanders may become functionally impaired. Guided by the fact that senile plaques in AD frequently involved not only extracellular Aβ deposits but also dystrophic neurites and reactive astrocytes (28), we analyzed for alterations in synaptophysin and glial fibrillary acidic protein immunostaining. It was interesting to find that regions of the brain which normally have an abundance of astrocytes, including regions of the corpus callosum overlying the CA1 and CA3 subregions of the hippocampus, showed detectable gliosis upon Aβ deposition (Fig. 3 *d*). The involved astrocytes became morphologically activated which gave the areas a denser appearance upon glial fibrillary acidic protein immunostaining.

Concomitant neuritic involvement in these extracellular

deposits was suggested by a marked increase in synaptophysin immunostaining within regions which normally have a basal level of immunoreactivity, such as areas immediately adjacent to the CA1 and CA3 neurons (Fig. 3 *e*); the focal increase in synaptophysin immunoreactivity is based upon comparisons of similar regions in nontransgenic animals. Since the increased immunostaining precisely overlapped the Aβ deposits, it may represent the entrapment of underlying neurites (Fig. 3 *m*). Interestingly, the presence of synaptophysin immunoreactivity has also been demonstrated in AD plaques (29). Further evidence for actual impairment of neurites was obtained using Bielschowsky's stain which highlighted the fine structure of the neurites extending from the hippocampal neurons. Unlike those not underlying the deposits, which have fine hair-like structures, many of those found underlying the Aβ deposits appeared blunted with knob-like structures at their ends (Fig. 3 *k*, *arrows*). This observation may very well suggest impairment of bystanding neurons which possibly, result in a second level of cell death. We are currently attempting to better define the extracellular deposition and its associated damages by thioflavin S staining and electron microscopy.

Discussion

Transgenic animals can be particularly useful in defining biochemical mechanisms using pathological changes as a functional readout. Indeed, this technology has been applied to better understand the etiology of AD. To date, no single model fully recreates the entire spectrum of pathological changes that occur in AD, although each has its own value for dissecting particular aspects of the pathogenesis of this insidious disease. A recent report suggests that expression of a human mutant *APP* gene in mice can reproduce some of the key pathological features of AD, including extracellular Aβ deposition, dystrophic neuritic components, gliosis and loss of synaptic density (30). Surprisingly, despite the fact that the selected transcriptional promoter is highly active in neuronal cells during embryonic development (30, 31), the lack of any evidence of cell death even in aged mice with abundant Aβ deposition is puzzling. This latter finding may well be interpreted to suggest that extracellular Aβ plaques are not involved in neuronal cell death in AD brains.

This study was directed at using the transgenic technology to test a crucial hypothesis regarding the pathophysiological role of Aβ in AD: is Aβ neurotoxic in an in vivo setting and, if so, how is the neurotoxicity manifested? Although targeting expression of Aβ intracellularly may seem unorthodox, the rationale behind our approach is supported by mounting evidence from several laboratories. First, not only is the endosomal/lysosomal pathway involved in the processing of Aβ precursors (1–3), a separate processing pathway occurring in the Golgi complex has recently been shown to result in intracellular accumulation of Aβ (5). Second, Aβ immunoreactivity has been demonstrated inside neuronal cells from multiple species, including humans, monkeys, dogs, and rabbits (4–6, 32). In fact, in a recent analysis of brains from aged primates, it was observed that the nonfibrillar Aβ peptide exists intracellularly within neurons before the appearance of extracellular deposits of Aβ (32). Third, several groups have shown that Aβ is associated with a majority of intraneuronal neurofibrillary tangles (33, 34). Fourth, Aβ can be directly immunoprecipitated from cell extracts of transformed human neuronal cell lines (4). Fifth, it has been reported using transient transfection assays that intracellular expression of amyloidogenic fragments results in formation of amyloid-like fibrils which suffice to kill cells (35, 36) and that intracellular Aβ aggregates stimulate further accumulation of amyloidogenic fragments of APP (6).

Having demonstrated that intracellular accumulation of Aβ would suffice to induce neuronal cell death (11), we proceeded to determine the molecular events associated with this cell death. We found that Aβ can activate the p53 dependent apoptotic pathway and that extracellular deposition of Aβ occurs secondarily to neuronal cell death. Furthermore, we provide ultrastructural evidence that the cell death is consistent with an apoptotic pathway. We also observed that if the extent of deposition is sufficiently abundant, perhaps a reflection of the number of initial cells involved, the surrounding neurons may as a consequence also be recruited into the pool of dying cells. This series of findings may offer new insights regarding the etiology of human AD. These observations prompted us to evaluate brain tissues from human AD cadavers where we found that neurons expressing Aβ intracellularly also succumbed to apoptosis (our unpublished results). It is interesting to note that Aβ has been shown to be generated by distinct processing pathways inside the cell (5) and that it remains obscure which of these pathways is responsible for the pathological consequences leading to AD.

A surprising observation with the Aβ transgenic mice is that despite abundant transgene expression throughout the brain, only specific regions including the cerebral cortex, hippocampus, amygdala and thalamus, showed Aβ-induced cell death. This finding agrees well with observations in human AD that not every brain region is equally involved. Significantly, even within a susceptible region, such as the neocortex, only focal areas which varied from mouse to mouse showed Aβ accumulation and cell death. This may imply that Aβ is normally unstable in neurons from any part of the brain, but that under certain conditions it may be stabilized in specific brain regions. It appears that Aβ needs to accumulate to a threshold level to activate the p53 dependent apoptotic pathway. This suggestion implies that "cofactors" are required beyond genetic predisposition and that the Aβ transgenic mice may be useful for identifying such cofactors.

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