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Short Communication

Evidence of Oxidative Stress and *in Vivo* Neurotoxicity of β-Amyloid in a Transgenic Mouse Model of Alzheimer's Disease

A Chronic Oxidative Paradigm for Testing Antioxidant Therapies in Vivo

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Increased expression of antioxidant enzymes and heat-shock proteins are key markers of oxidative stress. Such proteins are abnormally present within the neuropathological lesions of Alzheimer's disease (AD), suggesting that oxidative stress may play significant but yet undefined roles in this disorder. To gain further insight into the role of oxidative stress in AD, we studied the expression of CuZn superoxide dismutase (SOD) and hemoxygenase-1 (HO-1), two established markers of oxidative stress, in a transgenic mouse model of AD. Immunohistochemistry with anti-SOD and anti-HO-1 antibodies revealed a very pronounced increase of these proteins only in aged transgene-positive mice. Interestingly, the distribution of the oxidative burden was largely overlapping with dystrophic neuritic elements in the mice as highlighted with anti-ubiquitin antibodies. Because the most conspicuous alterations were identified around amyloid (Aβ) deposits, our results provide strong support for the hypothesis that Aβ is neurotoxic *in vivo* and that such toxicity is mediated by free radicals. To obtain additional experimental evidence for such an interpretation (ie, a cause-effect relationship between Aβ and oxidative neurotoxicity), PC12 cells were exposed to increasing concentrations of Aβ or to oxidative stress. In agreement with the *in vivo* findings, either treatment caused marked induction of SOD or HO-1 in a dose-dependent fashion. These results validate the transgenic approach for the study of oxidative stress in AD and for the evaluation of antioxidant therapies *in vivo*. (Am J Pathol 1998, 152:871–877)

Recent advances in Alzheimer's disease (AD) stem from the study of a 40/43-amino-acid peptide called the amyloid β protein (Aβ).[1] This protein derives from a much larger precursor, amyloid precursor protein (APP), encoded by a gene in chromosome 21.[2,3] Deposits of Aβ are one of the most distinctive features of AD and occur mainly within senile plaques and within cerebral and meningeal blood vessels.[4] Similar amyloid deposits develop in older patients with Down syndrome and to a much lesser extent in normal aged individuals.[5]

Several independent lines of investigation have now converged to suggest that oxidative stress may be important in the pathogenesis of AD.[6] One line of evidence is the identification of markers of oxidation such as antioxidant enzymes,[7] heat-shock proteins (HSPs),[8] free carbonyls,[9] and peroxinitration[10] within the neuropathological lesions of AD. A second, but equally important, body of data suggests that the neurotoxic properties of Aβ[11] are mediated by oxygen free radicals.[12,13] This information, however, does not resolve the issue of whether oxidative stress is a cause or a consequence of the disorder. Most importantly, the bulk of the evidence for a neurotoxic role of Aβ comes from *in vitro* data, and

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whether such toxicity exists *in vivo* has been questioned in several reports.[14–18]

The recent development of transgenic models of AD provides a unique opportunity to gain insight into Aβ-induced oxidative neurotoxicity and the role of the stress response. As a first step to validate such an approach for the study of free radical oxidations, we examined whether the expression of CuZn superoxide dismutase (CuZnSOD) and heme-oxygenase-1 (HO-1), two key markers of oxidative stress, is abnormal in brain sections from a transgenic model of AD.[19] Sections were also immunostained with anti-ubiquitin antibodies, as immunolocalization of this protein has been used as a marker of both free-radical-mediated cytotoxicity and dystrophic neurites in AD.[20–24] We used mice overexpressing a mutant APP transgene with a mutation found in a large Swedish family with early-onset AD.[19] As revealed by standard immunohistochemistry, the expression of the indicated oxidation markers provided a striking contrast between transgene positive (Tg^+) and transgene negative (Tg^-) mice. Our results offer a remarkable parallel with earlier observations in AD brains and previous *in vitro* studies that implicate Aβ in oxidative neurotoxicity.

Materials and Methods

Immunohistochemistry

Brain tissue was obtained from aged Tg^+ mice (n = 3; age, 21 to 25 months), aged Tg^- littermates (n = 3; age, 21 to 27 months), and two young (age, 4 months) Tg^+ mice. The younger mice were included to determine whether any abnormal expression of markers of oxidative stress occurs before the onset of amyloid accumulation. The tissue was fixed in 4% paraformaldehyde for 16 hours, embedded in paraffin, and sectioned as per routine histological procedures.

Immunohistochemistry was performed using a standard method.[25] For detection of oxidative stress, we used polyclonal antibodies directed against CuZnSOD (Binding Site, San Diego, CA) and HO-1 (StressGen, British Columbia, Canada) at 1:500 dilutions. For these antibodies, the following controls were included: 1) immunostaining of tissue sections obtained from two neuropathologically confirmed cases of AD and two neuropathologically and intellectually intact age-matched control individuals, 2) immunostaining of sections from one AD case and one aged Tg^+ mouse with the primary antibodies after absorption of the antisera with the corresponding purified enzymes,[7] and 3) immunostaining of one aged Tg^+ mouse and one AD case with nonimmune rabbit serum. The antibodies also recognized the appropriate molecular weight species in Western blots, as described and illustrated in subsequent sections of this paper.

In addition, tissue sections from two aged Tg^+ and two aged Tg^- mice were immunostained with a polyclonal rabbit anti-ubiquitin antibody (Dako Corp., Carpinteria, CA). Immunoreactivity for ubiquitin can offer important additional information as this small HSP is known to form conjugates with certain stress-altered proteins[20,21] (ie, oxidative toxicity), and it is an established marker of dystrophic neurites in AD.[22–24] Controls for the anti-ubiquitin antibody included immunostaining of one of the AD cases and one Tg^+ mouse with primary antibody that had been absorbed with ubiquitin.

Deposits of amyloid in the brains of older mice and their absence in younger mice have previously been reported[19] but were again verified using a monoclonal antibody to Aβ as described.[19,26]

Cell Culture Experiments

To confirm a cause and effect relationship between Aβ and the induction of CuZnSOD and HO-1 (ie, oxidative stress), cultured PC12 cells were exposed to increasing concentrations of Aβ. The expression of the stress indicators after exposure to Aβ was examined in Western blots and was compared with that induced by a bona fide oxidative lesion. As the same antibodies were used in both Western blots and immunohistochemistry, these experiments also served the purpose of verifying the specific reactivities of the detecting antibodies used for the tissue stains. PC12 cells were grown in serum-free Dulbecco's modified Eagle's medium supplemented with 5 μg/ml insulin, 20 μmol/L progesterone, 100 μg/ml transferrin, 40 μmol/L selenium, and 100 μmol/L putrescine. Cells were exposed to various concentrations of Aβ25–35, the actively toxic fragment of Aβ,[11] or to a 50 μmol/L concentration of a control scrambled sequence KSGNMLGIIAG for 24 hours. The forward and scrambled peptides were obtained from Research Genetics (Huntsville, AL) using identical methods of synthesis for both sequences. Oxidative damage was produced by incubating the cells with the CuZnSOD inhibitor diethylthiocarbamic acid (DDTC). Inhibition of CuZnSOD by DDTC is a well established model of oxidative injury that has been used previously to induce death of various neuronal cell types.[27,28] The cells were treated for 24 hours, washed with serum-free medium, and collected by scraping. Cells were lysed on PBS with protease inhibitors (Complete, Boehringer Mahnheim, Indianapolis, IN), and 20 μg of protein/well were electrophoresed in SDS-polyacrylamide gels[29] (12% polyacrylamide for detection of CUZnSOD and 10% for HO-1). Western blots[24] were immunostained with the same antibodies as used for immunohistochemistry and detected by a commercial chemiluminescence kit as described[26] (Amersham, Arlington Heights, IL). Anti-SOD was used at 1:1000 and HO-1 at 1:2000.

Results

Immunohistochemistry

Immunohistochemistry for the indicated markers of oxidation, CuZnSOD and HO-1, provided a striking contrast between aged Tg^+ and Tg^- mice (Figure 1). The pattern of reactivity in aged Tg^+ mice was similar (but not identical) to that observed in AD[7,8] and is consistent with widespread oxidative injury.

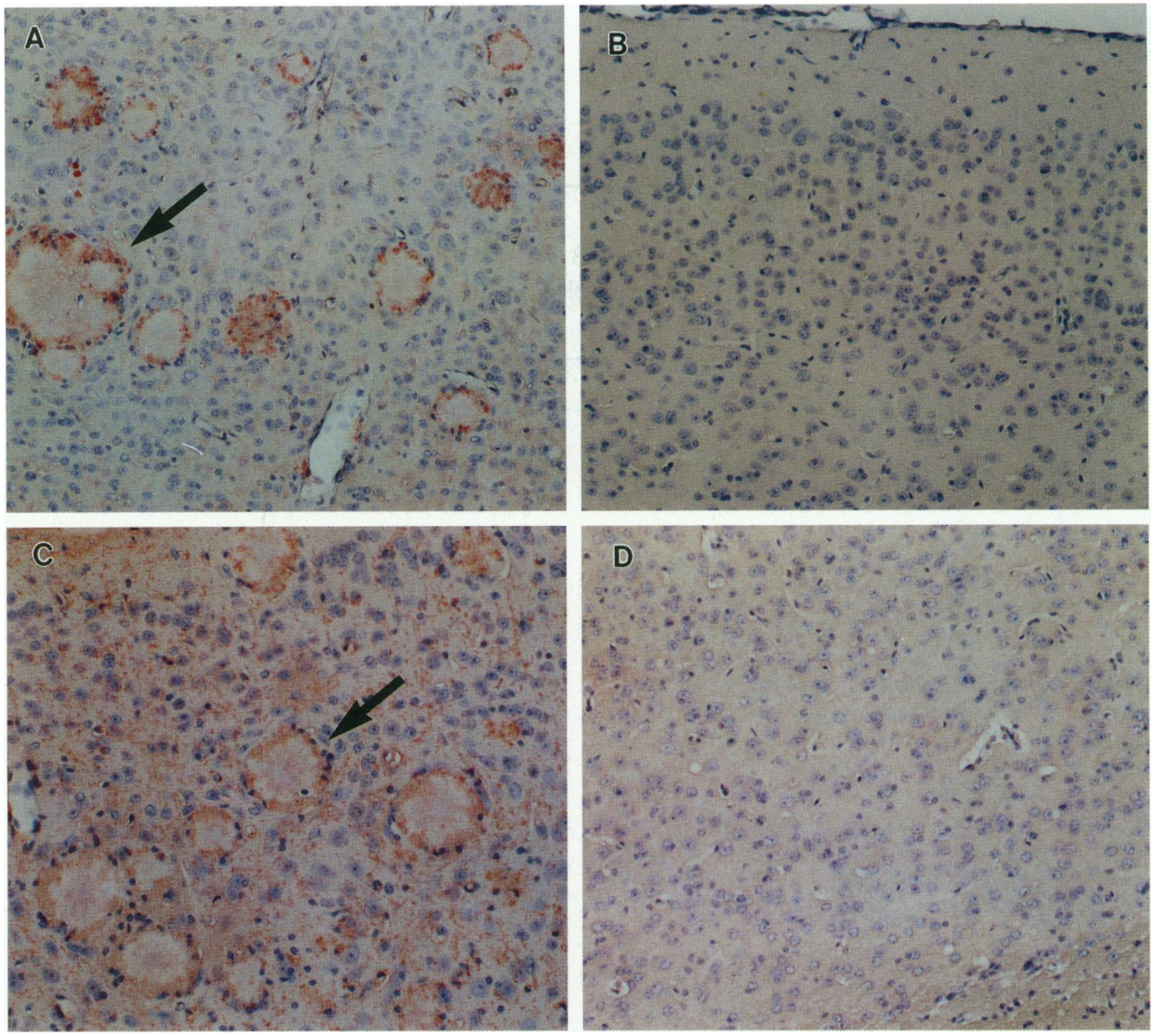

Figure 1. Immunostaining with anti-CuZnSOD (**A** and **B**) and anti-HO-1 (**C** and **D**) antibodies showed remarkable differences between TR^+ (**A** and **C**) and TR^- mice (**B** and **D**). Virtually every amyloid deposit in the mice was surrounded by strong immunoreactive deposits detected with both antibodies (**arrows**). Note fine granular deposits in **C**, immunoreactive with anti-HO-1 antibodies, within normal-appearing neuropil between the plaques. These fine granules were detectable only in TR^+ mice. Such intervening areas of normal-appearing neuropil were also devoid of reactivity with anti-Aβ (4G8) antibodies (not shown). Magnification, ×500.

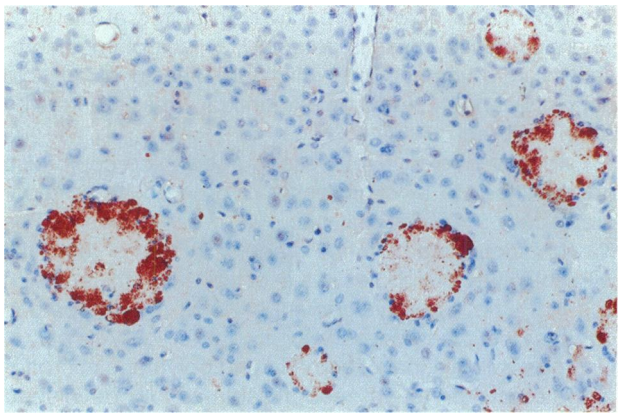

Figure 2. Immunostaining with anti-ubiquitin antibodies showed very strong labeling of granular structures around plaques, reminiscent of previous observations in AD brains. In contrast to AD brain, however, neurofibrillary tangles or neuropil threads were not present. The reported alterations were seen exclusively in aged Tg^+ mice. Magnification, ×1000.

Brain cortical areas in aged Tg^+ mice exhibited very strong and extensive reactivity for markers of oxidative stress. The staining was predominantly seen around amyloid deposits (Figure 1, A and C). Neuronal soma and amyloid remained unlabeled. Interestingly, anti-HO-1 antibodies highlighted subtle fine granular immunoreactive deposits in some neuropil areas of two (of three) aged Tg^+ mice (Figure 1C). Because these fine granules were present in cortical areas devoid of amyloid plaques, the oxidative burden also appears to extend (to a much lesser degree) beyond the visible amyloid deposits. With anti-CuZnSOD antibodies, the reactivity was confined exclusively to peri-plaque areas. With both antibodies, a conspicuous difference between Tg^+ mice and AD brain was the absence of neuronal staining in the mice. Sections from Tg^- mice were consistently unreactive with both antibodies (Figure 1, B and D).

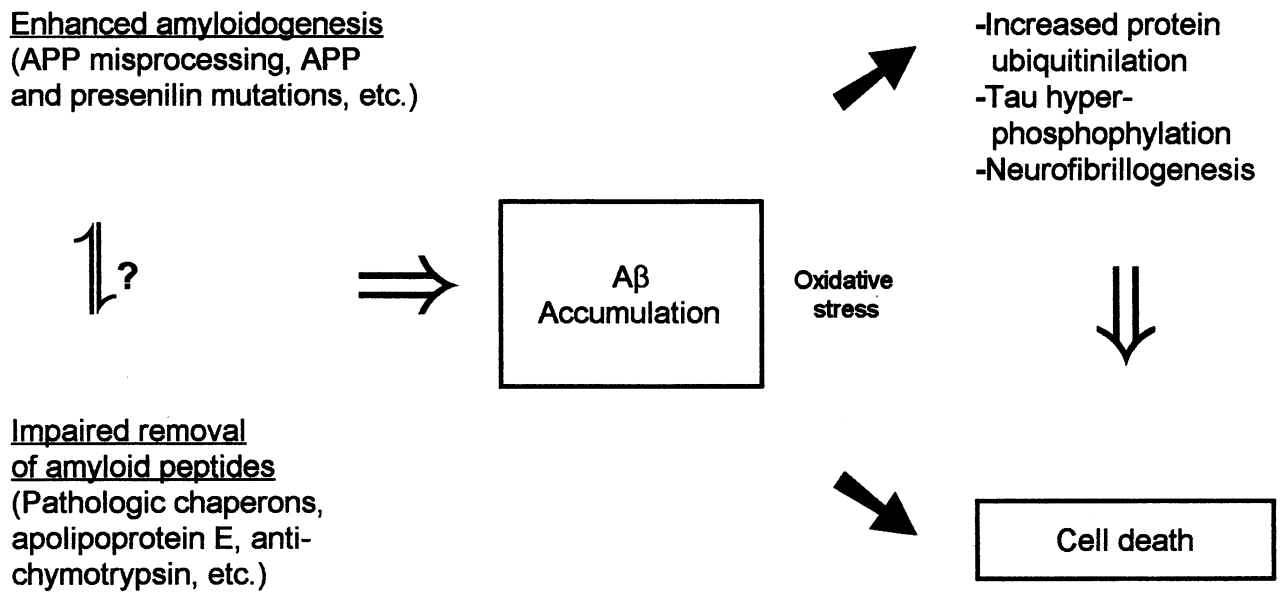

Figure 3. Proposed roles of oxidative stress in AD pathogenesis. As referenced in the text, the pathways indicated by **solid arrows** are supported by extensive experimental evidence.

With anti-ubiquitin antibodies, there was very strong labeling of granular peri-plaque dystrophic structures around virtually every amyloid deposit in aged Tg^+ mice (Figure 2). There was an extensive overlap between the markers of oxidation and the ubiquitin immunoreactive structures. This overlap strongly supports the pathogenic sequence proposed by many investigators, ie, that amyloid causes oxidative stress and that this modality of injury accounts for the development of dystrophic neurites (Figure 3).

Control brains with AD and normal human brains immunostained with anti-CuZnSOD, anti-HO-1, and anti-ubiquitin showed the expected pattern of reactivity as previously reported.[7,8,20] Absorption of the primary antibodies with the corresponding purified antigens completely eliminated the immunoreactivity in mice and human brain. Appropriate lack of immunostaining was also verified with control nonimmune rabbit serum.

Sections from the two young control Tg^+ mice did not show any detectable staining with anti-CuZnSOD, anti-HO-1, or anti-ubiquitin antibodies (Figure 4). As shown in previous studies,[19] no amyloid deposits were found in these younger (4-month-old) Tg^+ mice (Figure 4).

Cell Culture Experiments

A fraction of PC12 cells exposed to Aβ25–35 underwent degeneration and death as previously reported by us[28] and several other investigators. Western blots from cell lysates exposed to increasing concentrations of Aβ25–35 showed a clear-cut induction of SOD and HO-1 (Figure 5). This induction occurred with a conspicuous dose-response pattern (Figure 5). The results are in qualitative agreement with previously published reports showing that oxidative lesions are produced after exposure of cells to Aβ (see Discussion). Identical protein species were induced, also in a dose-response fashion by a bona fide oxidative injury with DDTC (Figure 5). As it can be appreciated in the figure, both antibodies specifically identified the appropriate molecular weight species.

Discussion

The focus of the current investigation was to validate the transgenic approach for the study of oxidative stress in AD. At this time, no attempts were made at elucidating the molecular mechanisms involved in the reported abnormalities. Several conclusions, however, can be reached from the presented data. The results support a relationship between oxidative stress and Aβ-mediated neurotoxicity *in vivo*. Such a connection is suggested by the following findings: 1) marked accentuation of immunoreactivity for SOD and HO-1 around amyloid deposits, 2) presence of ubiquitin-reactive structures at the sites where oxidative stress is detected, 3) lack of immunoreactivity in younger mice (which are devoid of amyloid deposits), and 4) induction of identical stress markers by Aβ in cultured cells.

Evidence of oxidative stress in aged mice could also be detected, to a much lesser degree, in areas topographically unrelated to senile plaque-like deposits. This was characterized by a fine granular pattern of reactivity with anti-HO-1 antibodies in two of three Tg^+ mice, which suggests that the development of senile plaque pathology may not be necessary for oxidative injury to occur. One possibility is that oligomeric and polymeric microaggregates of Aβ, not yet recognizable as fully developed plaques, are sufficient to produce a minimal but yet detectable induction of the stress response. Another possibility may involve APP overexpression, although this is unlikely due to the absence of staining in younger mice.

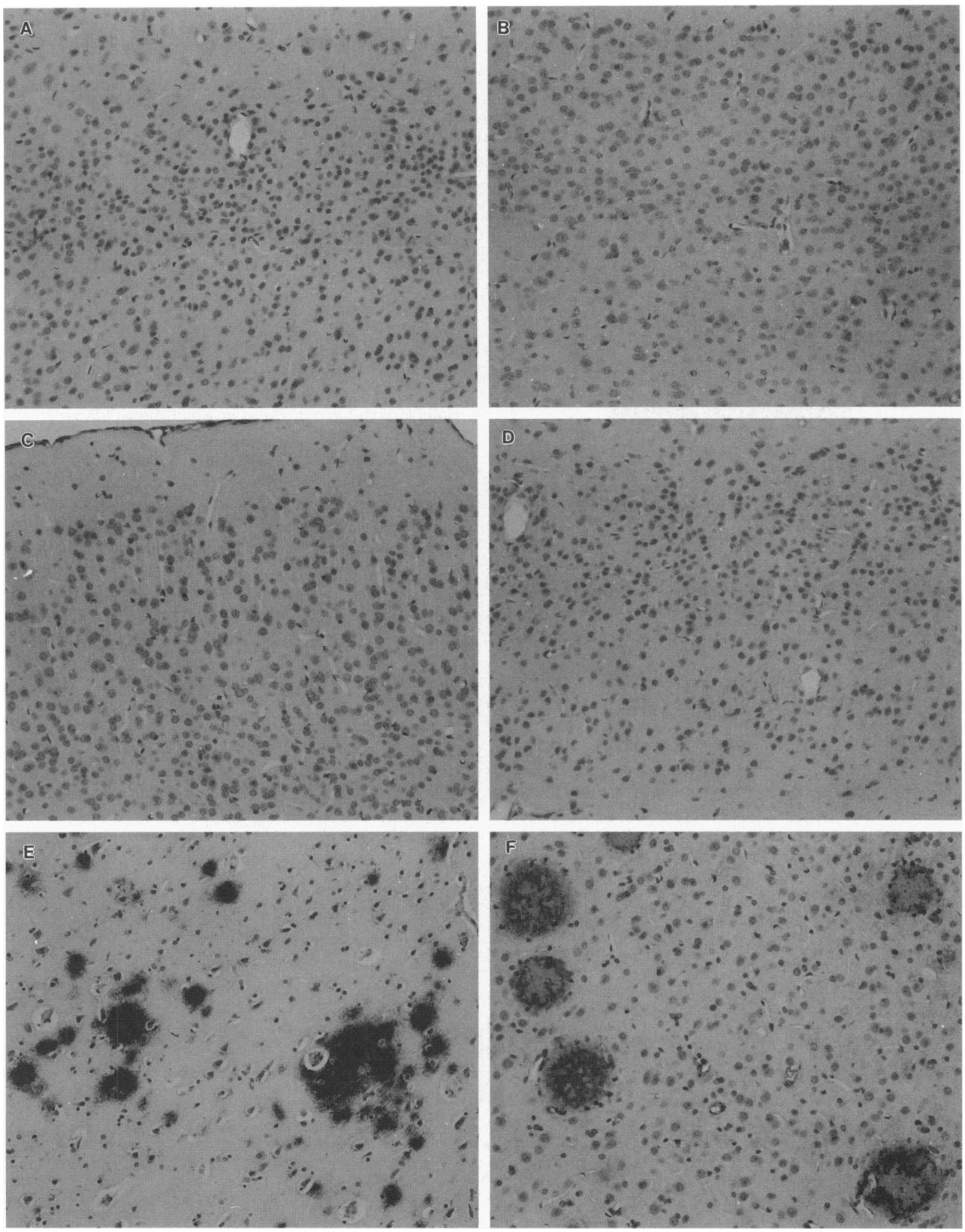

Figure 4. Representative areas from 4-month-old Tg^+ mice illustrating complete lack of immunoreactivity after immunostaining with antibodies against Aβ (**A**), CuZnSOD (**B**), HO-1 (**C**), or ubiquitin (**D**). **E** and **F** depict representative areas of AD and aged Tg^+ mice cortex, respectively, stained with antibodies against Aβ (4G8 antibody). Magnification, ×500 (**A** to **D** and **G**) and ×200 (**F**).

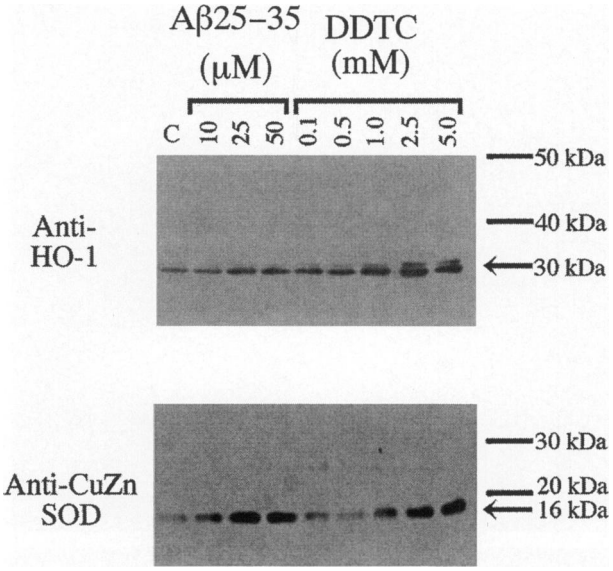

Figure 5. Computer scans of Western blots of PC12 cells treated with the indicated concentrations of Aβ25–35 (**left lanes**) or with the CuZnSOD inhibitor DDTC (**right lanes**). Both treatment modalities caused increased expression of HO-1 (**upper gel**) and CuZnSOD (**lower gel**) as detected by the same antibodies employed in the tissue immunostains. The first **lane** on each gel are control cells treated with 50 μmol/L scrambled peptide.

Confirmation of these interpretations in subsequent studies may help explain the apparent lack of correlation between cell loss and senile plaques in human brain.[30–22]

Taken together, the results are in agreement with previous *in vitro* studies linking oxidative stress to Aβ toxicity.[12,13,33–39] In *in vitro* studies, exposure of cells to Aβ resulted in marked oxidative injury, including lipid peroxidation,[13,28] protein carbonyl formation,[13] enzyme inactivation,[13,35,36] mitochondrial DNA damage,[37] and induction of stress-related alterations including ubiquitination of cytoskeletal proteins.[38,39] As many of these lesions could be prevented by antioxidant or anti-amyloidogenic agents (ie, *N*-t-butyl-α-phenylnitrone,[28] vitamin E,[33] β-sheet breakers,[40] and melatonin[28,37]), it will be particularly important to determine whether treatment of these mice with similar compounds could ameliorate the oxidative burden or the reported behavioral abnormalities.

The molecular mechanisms involved in the oxidative stress response induced by Aβ *in vivo* will be an interesting subject for future investigations. In *in vitro* systems, cells exposed to Aβ generate increased H_2O_2 that in the presence of transition metals gives rise to damaging free radicals.[12] Free radical generation by Aβ may also be enhanced by binding of amyloid to cell surface receptors[41] or by receptor-independent mechanisms. Aβ is known to affect intracellular Ca^{2+} homeostasis,[42] which causes a number of deleterious changes, including activation of proteases and NADPH oxidase, which in turn causes increases in peroxide production.[43] Elevated intracellular Ca^{2+} also causes increased NO production through activation of calmodulin-dependent nitric oxide synthase.[10] Reaction of NO with the superoxide radical O^{2-} generates the highly reactive peroxynitrate, which was shown to be as damaging to cell structures as hydroxyl radicals. In this regard, widespread peroxynitration of proteins has recently been demonstrated in AD brains.[10,45]

Recent reports have suggested that Aβ may not be toxic *in vivo*.[14–18] The information generated in this study, however, suggests that this is not the case. Using this transgenic model, a recent report concluded that "Aβ is not acutely neurotoxic," mostly because neuronal loss was undetectable in the transgenics.[18] However, the absence of neuronal death in mice in the face of the evidence of oxidation may simply reflect a greater degree of resistance to oxidative injury in mice compared with humans.

Among the most important unresolved issues regarding the role of oxidation and AD is whether oxidative stress triggers or follows the sequence of events that would culminate with the death of neurons. It has recently been proposed that oxidative stress would trigger a pathological vicious cycle in which amyloidogenic processing of APP would be further enhanced by oxidative stress[46–48]; thus, misprocessing of APP would lead to more oxidations and again to more amyloid production. Whether this self-sustaining cycle is indeed involved in AD remains speculative.

In conclusion, the study of oxidative stress in AD as well as the evaluation of experimental antioxidant therapies have been hampered by the vexing level of complexity of the stress response in tissues. Induction of HSPs in glial cells, for example, is followed by export of the proteins into adjacent axons so that compartments that are distant from the neuronal soma can be effectively protected.[49] Such intricate interactions are ideally studied in appropriate *in vivo* paradigms. The data presented here suggest that available transgenic models may be explored as viable alternatives for the study of oxidative stress in AD. This strategy will also be of use in testing therapies aimed at reducing the overall oxidative burden associated with the disease.

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