American Journal of Pathology, Vol. 140, No. 3, March 1992

Immunohistochemical Evidence of Antioxidant Stress in Alzheimer's Disease

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Membrane and cytoskeletal structures are known targets of oxidative injury. Brains from patients with Alzheimer's disease have cytoskeletal abnormalities and platelet and possible neuronal membrane lesions. The authors have recently demonstrated that superoxide anion is a powerful inducer of heat-shock protein synthesis, and have also shown that in response to oxidative stress or hyperthermia, intracellular levels of antioxidant enzymes increase to several folds. Whether the aforementioned mechanisms play a role in Alzheimer's disease has been suggested but is not totally established. While exploring this possibility, tissue sections from five brains with Alzheimer's disease and five neuropathologically normal age-matched controls were immunostained with polyclonal antibodies against superoxide dismutase (CuZn- and Mn- forms) and catalase. A standard avidin-biotin-peroxidase method was used for antigen detection. A subgroup of neurofibrillary tangles (15–25%) and senile plaques (50%) showed immunoreactivity for both enzymes with a staining pattern similar (but not identical) to that usually observed with antibodies against ubiquitin. Senile plaques displayed a granular pattern of immunostaining. Amyloid cores in mature classical plaques remained unstained. In addition, occasional elements with features consistent with reactive glial cells were strongly immunostained. Tangle-free neurons in both diseased and control brains showed weak to absent intracytoplasmic immunoreactivity. The immunoreactivity was totally abolished by preincubation of the primary antibodies with the corresponding purified antigens. These findings support the hypothesis that oxidative stress may be involved in the pathogenesis of Alzheimer's disease. (Am J Pathol 1992, 140:621–628)

Exposure of cells to hyperthermia and various other forms of stress is followed by the induction or preferential synthesis of characteristic sets of polypeptides termed heat-shock proteins (HSP).[1–4] Inducers of HSP synthesis can be as varied as glutathione depleters, viruses, heavy metals, reoxygenation after anoxia, or peroxides.[1,5–8] Despite this diversity, most inducers seem to cause injury in a pattern consistent with free radical reactions.[9–11]

Data from our own studies[10,12] and from other laboratories[11] have shown that free-radicals themselves are powerful inducers of HSP synthesis. We have previously documented that hyperthermia is followed by a pronounced rise in antioxidant enzyme levels as part of the overall cell response to stress.[9]

The function of HSPs is not totally understood. However, their highly conserved nature has long suggested a significant role in the cellular defense against various forms of damage. Cytoskeletal structures are among the primary targets of injury caused by HSP inducers or oxygen-free radicals.[13,14] Under these conditions, some HSPs bind avidly to cytoskeletal proteins[13,14]; these associations are attended by development of some degree of cytoskeletal tolerance to subsequent injury.[15]

Another significant facet of the overall cellular adaptation to stress is ascribed to a small HSP termed ubiquitin. Among a number of metabolic functions, this protein is part of an important proteolytic system aimed at ridding cells of abnormal proteins generated under stress.[16–19] Overwhelming this housekeeping capacity may lead, as we and others have suggested,[20–22] to the accumulation of ubiquitinilated protein conjugates in the form of cytoskeletal inclusions.

Accepted for publication September 20, 1991.

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Abnormal cytoskeletal accumulations known as neurofibrillary tangles (NFT) are one of the most prominent neuropathologic features of Alzheimer's disease.[23,24] Another such feature is the senile plaque, which are foci of neuropil degeneration containing amyloid and its precursors, dystrophic neuritic processes and microglial proliferation.[25] The possible involvement of oxidative or heat shock type of injury in the pathogenesis of the described lesions has been suggested[10,26–32] but not yet proven. During the course of investigating the aforementioned possibility, we immunostained brain sections from patients with Alzheimer's disease with antibodies against catalase and superoxide dismutase (SOD) (Mn- and CuZn- forms). Our results support the hypothesis that oxidative stress is involved, at least at some point, in the pathogenesis of Alzheimer's disease.

Materials and Methods

Antibodies

Anti-SOD (Mn- and CuZn- forms) and anticatalase antibodies were purchased from a commercial source (Binding Site, Birmingham, England). The specificities of the immunochemical reactions were confirmed by overnight incubation of each antibody with its corresponding purified antigen. SOD (CuZn- form) and catalase were purchased from Calbiochem (La Jolla, CA) and SOD (Mn- form) was obtained from Sigma (St. Louis, MO).

Additionally, we believed it was prudent to exclude crossreactivity of these antibodies with microtubules and neurofilaments, the main cytoskeletal components of the neuron. For this purpose, we purified calf-brain microtubules by three assembly-disassembly cycles according to the method of Borisi et al[33] and their identity confirmed by electron microscopy and the Western Blot method as we described in detail previously.[24] Neurofilaments were obtained from rat spinal cords by the axonal flotation method[24] and their identity was also confirmed by polyacrylamide gel electrophoresis and electron microscopy as we previously reported.[24] The antibodies were reacted with the aforementioned cytoskeletal preparations in dot-blot experiments using an alkaline phosphatase detection system.[34] Two μg of each cytoskeletal preparation and 2 μg of catalase, SOD Mn- or SOD CuZn- were placed on nitrocellulose sheets and immunoreacted with the corresponding primary antibodies as described.[24] The anti-beta-protein antibody was a monoclonal (4G8) raised against amino acids 17 to 28 of the beta-protein sequence. Full characterization of this antibody has previously been published.[35]

Immunochemistry and Tissue Samples

The immunostaining technique used in this study was the standard avidin-biotin-peroxidase method.[36] The primary antibody dilutions were as follows: 1:750 for both anti-SOD antibodies, 1:500 for anti-catalase, and 1:1000 for the anti-beta-protein antibody.

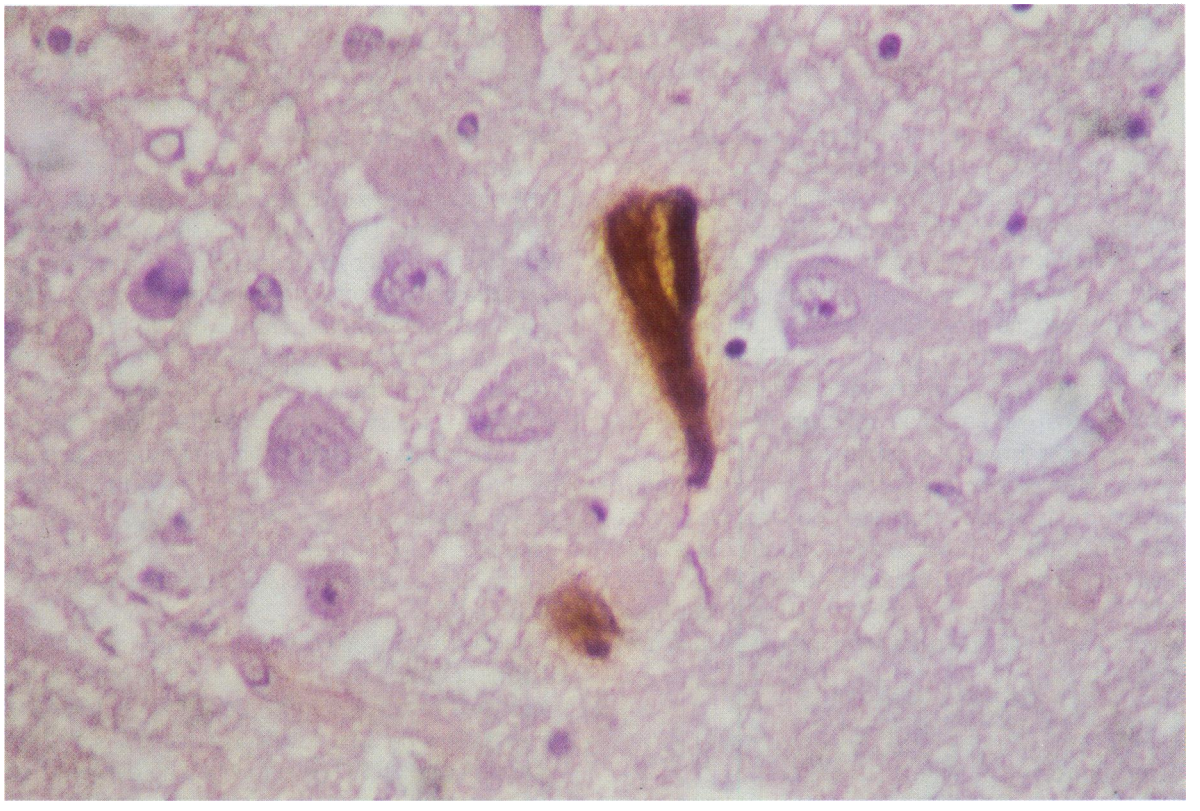
Paraformaldehyde-fixed hippocampi and adjacent parahippocampal gyri from five cases meeting the National Institutes of Health consensus criteria for the neuropathologic diagnosis of AD[37] were obtained for this study.

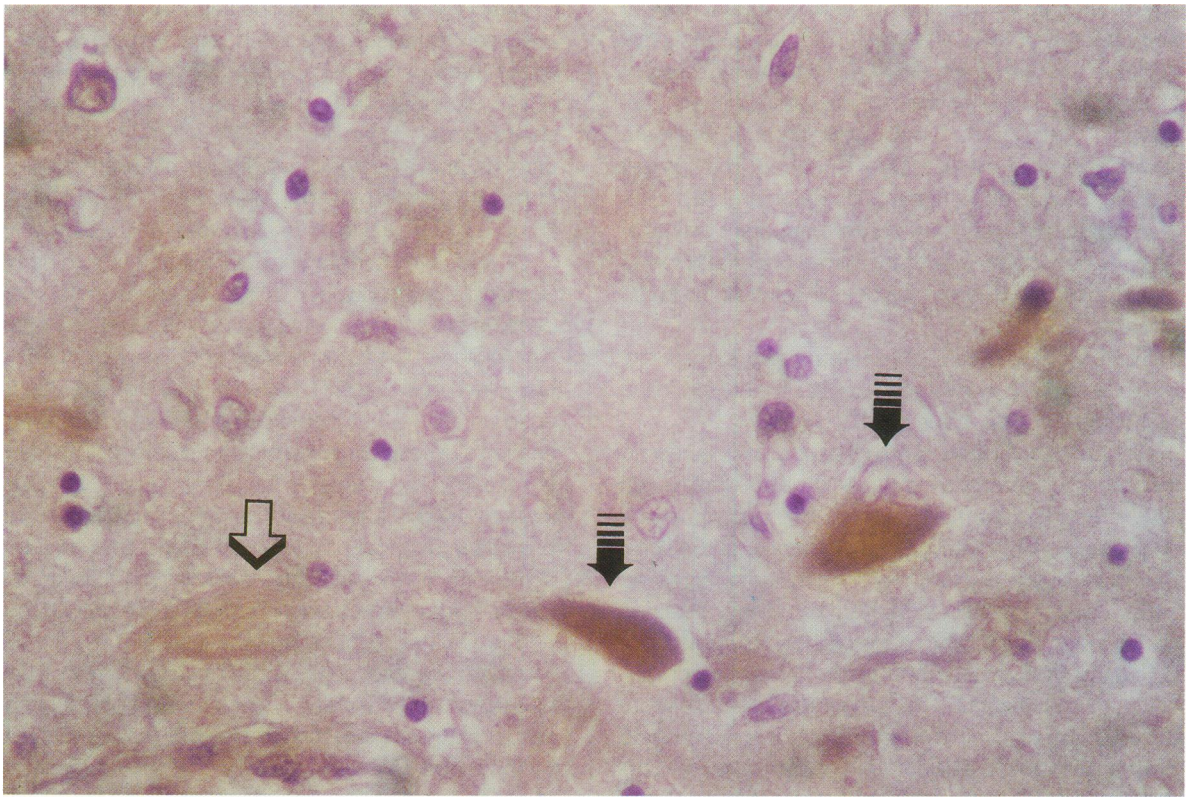
As control material, we selected five brains matched for age and postmortem interval obtained from intellectually intact patients who had died of non-neurological causes and showed absence of senile plaques and neurofibrillary tangles as determined by the Gros-Schultze version of the Bielschowsky method.[38] Controls for the technique included sections incubated with the corresponding nonimmune sera instead of the primary antibodies.

Results

Except for minor differences, the general pattern of immunoreactivity with all three antibodies was similar. A subset of NFTs was immunoreactive with the three antibodies in all cases of AD (Figures 1, 2). The number of immunoreactive NFTs ranged from 15% to 25% of all NFTs detected in these cases with silver impregnation by the Gros-Schultze version of the Bielschowsky method. Extracellular neurofibrillary tangles were unreactive with few exceptions showing only minimal staining. With all three antibodies, tangle-free neurons in all cases of AD and neurons in the five normal control brains showed weak to absent immunoreactivity. Sections from two of the cases with AD showed a higher density of immunoreactive NFT with anti-SOD and anti-catalase antibodies. These particular cases had been fixed for shorter periods, thus suggesting some degree of antigenic loss by tissue fixation in the remaining cases with AD. Trypsin digestion increased the intensity of immunoreactivity in two cases with AD, decreased it in one case, and af-

Figure 1. *Microphotograph depicting a strongly reactive neurofibrillary tangle with anti-SOD (CuZn-form) antibody. (Original magnification ×400, avidin-biotin-peroxidase).*
Figure 2. *Microphotograph showing immunoreactive intracellular neurofibrillary tangles with anti-SOD (Mn-form) antibody (interrupted arrows). An extracellular tangle is barely discernable (open arrow). (Original magnification ×400, avidin-biotin-peroxidase).*





fected no changes in immunostaining intensity in two cases.

Senile plaques showed a predominantly granular pattern of immunostaining which in instances resembled that obtained with anti-ubiquitin antibodies[39] (Figure 3). Immunoreactivity was present in approximately 50% of senile plaques detected by silver impregnation and by immunostaining with the anti-beta/A4 antibody (not shown). A small proportion of senile plaques were surrounded by immunoreactive cells with morphology corresponding to reactive glial cells. This was a finding more frequently observed with the anti-catalase antibody and anti-SOD Mn- antibody only rarely with the anti-SOD CuZn- antibody (Figures 4, 5). Amyloid cores in mature or burnt-out plaques remained unstained, although rarely, a peripheral immunoreactive rim surrounded the amyloid.

Negative controls (sections incubated with nonimmune sera instead of primary antibodies) were completely negative. Immunostaining of known positive sections with preabsorbed antibodies yielded negative results. The anti-SOD's and anti-catalase antibodies also failed to react with the cytoskeletal preparations in dot-blots.

Discussion

The presence of high levels of antioxidant enzymes in association with NFTs and senile plaques suggests that oxidative stress may be involved in the pathogenesis of these lesions. However, alternative explanations are conceivable. Since only a small proportion of lesions were immunoreactive, one possibility is that our findings just represent a late event in the pathogenesis of AD. Likewise, oxidative stress may become apparent only after neurons become metabolically altered (thus inefficient to manage normal oxidative loads) by a totally unrelated process.

Nevertheless, several observations suggest that oxidative stress may play more than a secondary role in the pathogenesis of the lesions of AD.

Subbarao and coinvestigators have shown increased *in vitro* lipid peroxidation in cortical tissue from brains with AD.[40] Their findings have recently been confirmed by another group of investigators (Andorn et al, personal communication). Zemlan et al[31] have found increased superoxide dismutase activity in fibroblasts obtained from patients with AD and suggested that the formation of paired helical filaments (one of the characteristic ultrastructural components of NFTs) might be free-radical mediated. Blass et al discovered that exposure of cultured cells to uncouplers of oxidative phosphorylation (i.e., oxidative stress) is followed by induction of ALZ-50 and paired helical filamentlike immunoreactivity.[29] Upholding these latter studies is a recent report of cytochrome-oxidase deficiency in platelets obtained from patients with AD.[41] In this connection, it should be recalled that in patients with AD, platelets[42,43] and possibly brain cells[44,45] have reported membrane abnormalities consistent with oxidative injury.

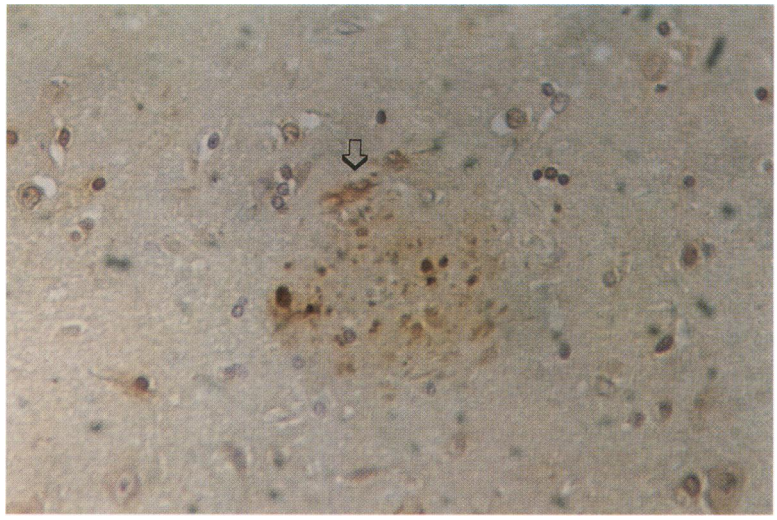
We have shown[9] that intracellular antioxidant enzyme activity increases to several folds after exposure of cells to hyperthermia. Under such conditions, there is profound disruption of cytoskeletal networks, accompanied by increased levels of protein ubiquitinilation.[13,14] These stress-induced changes parallel what we see in NFTs, i.e., abnormal cytoskeletal aggregates[23,24] which are targets for heavy ubiquitinilation.[46] However, the presence of ubiquitin-protein conjugates in NFTs[46] and in other cytoskeletal inclusions[47] has not universally been accepted as unequivocal evidence of stress-induced injury. Alternate processes, such as inhibited proteolysis, increased generation of aberrant proteins, saturation of the ubiquitin system, or a combination of these events, have all been proposed.[39]

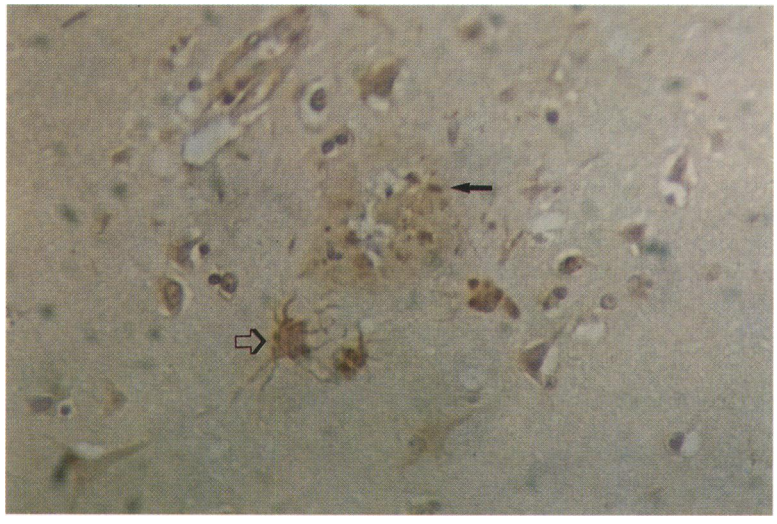
At this time, we do not have information as to the activity of SOD and catalase in our cases, although this important question is currently being explored. Previous investigations on SOD activity in erythrocytes obtained from patients with AD have reported no difference between samples from AD and control patients.[48]

Marklund et al examined the activities of SOD in several brain regions and reported "only very small differences" between the AD and the control group.[49] Review of the aforementioned investigators' data, however, shows modest increases in SOD activity in AD hippocampus and a "tendency to higher activities" with increasing age and in AD.[49] In view of our current findings, this issue may have to be re-examined more extensively.

At odds with our current data are recent studies by Somerville and coworkers.[30] These investigators reported significant reductions in CuZn-SOD mRNA in the CA1 region of the hippocampus of brains with AD. The aforementioned disparity, however, may be more apparent than real. It has been demonstrated that the enhanced synthesis of certain proteins during heat shock is not attended by changes in their encoding mRNA levels; instead, there is a conversion of free, translationally repressed mRNA to a polyribosome-bound, translationally active form.[50] Whether such mechanism controls antioxidant enzyme synthesis during stress is not known.

The CuZn-SOD gene is in the long arm of chromosome 21,[51] which is duplicated in Down syndrome. It has been proposed that the AD type of neuropathology observed in the aforementioned disorder may be caused by increased CuZn-SOD activity.[52–54] Both, increases or decreases in SOD activity can exacerbate production of free radicals.[53]





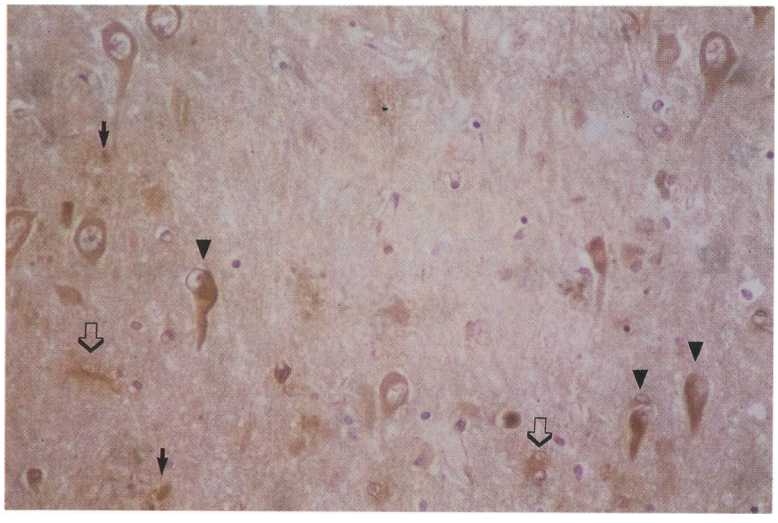


Figure 3. *Microphotograph of senile plaque containing "granular" immunoreactive deposits with anti-SOD (CuZn-form) antibody. Rare reactive glial cells were detected (open arrow). (Original magnification ×200, avidin-biotin-peroxidase).*

Figure 4. *Immunostaining with anti-catalase antibody show "mature" senile plaque with immunoreactive glial cells at periphery (open arrow) and "granular" deposits (solid arrow). Amyloid core remained unreactive. (Original magnification ×200, avidin-biotin-peroxidase).*

Figure 5. *Representative overall pattern of immunoreactivity with anti-catalase antibody. Glial cells (open arrows), neurofibrillary tangles (arrowheads), and neuropil "granular" deposits (not observed in normal control brains, solid arrows) can be recognized. (Original magnification ×100, avidin-biotin-peroxidase).*

Another point of interest is the possible relationship between CuZn-SOD and the amyloid precursor protein (APP).[48] Current investigations suggest that synthesis of some APPs in brains with AD are increased.[55–58] The structural gene for CuZn-SOD is located between the gene encoding APP and the proto-oncogene ets-2.[59] Since genetic information may be read *en block* from DNA segments encompassing several genes,[60] it can

be envisioned that increased expression of SOD and APP may occur simultaneously under conditions of oxidative stress. Increased immunoreactivity for catalase (which gene is in a different location) would favor oxidative stress as the trigger of this response. The APP gene promoter contains a heat-shock element that might be activated by heat shock or oxidative stress.[61]

In conclusion, we demonstrated increased immunoreactivity for antioxidant enzymes in association with the neuropathologic lesions of AD. Since the immunoreactivity for these enzymes was limited to a small subgroup of lesions, oxidative stress may follow, rather than precede, development of AD-related neuropathology. Further investigations are needed to determine the role of unscheduled cellular oxidations in the pathogenesis of this disease.

Note Added in Proof

Two groups of investigators showed heat-shock proteins in the AD brain,[62,63] which confirmed our initial preliminary report,[64] and supports the hypothesis proposed in this article. In December 1991, Smith et al reported increased protein oxidation in enzyme dysfunction in the normal aging brain and AD.[65]

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