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Short Communication

Heme Oxygenase-1 is Associated with the Neurofibrillary Pathology of Alzheimer's Disease

Mark A. Smith,* R. Krishnan Kutty,† Peggy L. Richey,* Shi-Du Yan,‡ David Stern,‡ Gerald J. Chader,† Barbara Wiggert,† Robert B. Petersen,* and George Perry*

From the Institute of Pathology, Case Western Reserve University, Cleveland, Ohio; Laboratory of Retinal Cell Biology and Molecular Biology,† National Eye Institute, National Institutes of Health, Bethesda, Maryland; and Department of Physiology,‡ Columbia University, College of Physicians and Surgeons, New York, New York*

Heme oxygenase-1 is an important enzyme that degrades heme, a pro-oxidant, leading to the formation of antioxidant molecules. In this study we demonstrate by immunocytochemistry close association of heme oxygenase-1 with Alzheimer neurofibrillary pathology and with the neurofibrillary tangles found in progressive supranuclear palsy and subacute sclerosing panencephalitis. In Alzheimer's disease, using two different rabbit antisera against heme oxygenase-1 protein, we localized, using immunocytochemical methods, heme oxygenase-1 to neurofibrillary tangles, senile plaque neurites, granulovacuolar degeneration, and neuropil threads. Only light background staining was seen in young controls and sporadic lesion-related immunoreactivity in age-matched controls. The increase in heme oxygenase-1 protein in association with the neurofibrillary pathology of Alzheimer's disease and other diseases characterized by neurofibrillary tangles supports the notion that the generation of free radicals and oxidative stress plays a role in the pathogenesis of neurofibrillary pathology. (Am J Pathol 1994, 145:42–47)

Heme oxygenase (HO) is a microsomal enzyme that oxidatively cleaves heme, a pro-oxidant, to produce biliverdin and carbon monoxide.[1] Biliverdin is converted to bilirubin, a potent antioxidant,[2] and within the brain carbon monoxide is suggested to act as a neurotransmitter.[3] HO consists of two homologous isozymes,[4] an inducible HO-1 and a constitutively produced HO-2.[5–7] HO-1 is induced by a wide variety of stimuli including conditions of oxidative stress and heat shock.[1,8–10]

Within the brain, the majority of HO activity is attributed to the HO-2 isozyme.[11] The expression of HO-1 is very low in normal brain but markedly increases after heat shock or glutathione depletion.[11–14] In the normal rat brain, HO-1 is present in select neuronal and nonneuronal cell populations in forebrain, diencephalon, cerebellum, and brain stem.[13] After heat shock, increased HO-1 is seen in glia throughout the brain, ependyma lining the ventricles of the brain, paraventricular nucleus, Purkinje cell layer of the cerebellum, and cochlear nucleus of the brainstem.[13]

Oxidative stress is implicated in a number of neurodegenerative diseases, including Alzheimer's disease.[15,16] Furthermore, the characteristic pathological lesions of Alzheimer's disease, senile plaques, and neurofibrillary tangles are associated with several heat shock proteins, including ubiquitin,[17,18] HSP27,[19] HSP70,[20] and αB-crystallin.[21] Based on this evidence, we used an immunocytochemical approach to investigate Alzheimer's disease brain for the presence of HO-1.

Materials and Methods

Tissue Section Preparation

Central nervous system tissue was obtained at postmortem from patients with histopathologically con-

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Address reprint requests to Dr. Mark A. Smith, Division of Neuropathology, Institute of Pathology, Case Western Reserve University, 2085 Adelbert Road, Cleveland, OH 44106.

firmed Alzheimer's disease, two other distinct conditions characterized by neurofibrillary tangles but not amyloid β-protein deposition (progressive supranuclear palsy and subacute sclerosing panencephalitis), and non-Alzheimer young and aged-matched controls. Tissue was fixed for 24 to 48 hours by immersion in formalin for all cases or methacarn (methanol:chloroform:acetic acid, 6:3:1) for Alzheimer's disease and control cases. Postfixed tissue was dehydrated through graded ethanol and xylene solutions and embedded in paraffin. Six-micron thick microtome sections were prepared.

Antibodies

Rabbit anti-HO-1 (SPA-895; Stressgen Biotechnologies Corporation, Victoria, Canada), rabbit anti-HO-1 peptide,[22] and rabbit anti-τ[23] were used as primary antisera in this study. A peptide, MERPQLDSMSQDLSEALKEATKEVHIRAEN, consisting of residues 1 to 30 from the sequence reported for rat HO-1[24] was used as antigen. The multiple antigenic peptide (MAP) system described by Posnett et al[25] was used for the production of rabbit antisera against the HO-1 peptide. Details of its preparation and characterization and its ability to react with human HO-1 have been described.[22]

Immunocytochemistry

After deparaffinization in xylene and rehydration through graded ethanol, endogenous peroxidase activity was inhibited by 20-minute incubation in 3% H_2O_2 and nonspecific protein binding sites blocked with 10% normal goat serum in Tris-buffered saline (150 mM Tris-HCl, 10 mM NaCl, pH 7.6). Immunostaining was by the peroxidase-antiperoxidase technique using 3,3′-diaminobenzidine as chromogen.[26] Adjacent sections were immunostained with anti-τ serum as previously described[23] to confirm the identity and location of pathological structures.

The antisera generated against HO-1-specific peptide[22] required a trypsin pretreatment of the section before the immunocytochemical protocol described above. In addition, for immunostaining with HO-1 antiserum (Stressgen Biotechnologies Corp.) we found that methacarn-fixed tissue was superior to formalin fixation requiring shorter incubation times and lower titers of antiserum.

Adsorption experiments were performed on anti-HO-1 to confirm the specificity of antibody binding. The immunostaining protocol was repeated, except here using adsorbed antiserum in parallel. Adsorbed antisera was generated by incubation of primary antisera with purified HO-1 protein (Stressgen Biotechnologies Corp.) diluted to a final concentration of 10 μg/ml for 3 hours at 37 C. Adsorption of anti-τ with HO-1 protein was also performed as a control against artifactual absorption.

Results

HO-1 Immunocytochemistry

Both HO-1 antisera (anti-HO-1 protein and anti-HO-1 peptide) strongly immunolabel the intracellular neurofibrillary pathology of Alzheimer's disease (Figure 1, A and C) including neurofibrillary tangles, senile plaque neurites, granulovacuolar degeneration, and neuropil threads. Staining adjacent tissue sections with anti-τ showed near total overlap in the immunostaining profiles of HO-1 and τ, except the former did not label extracellular neurofibrillary tangles (data not shown). The antiserum against the native protein gave more intense staining profiles of pathological structures than the antisera produced against HO-1 peptide. In addition, this latter antisera required partial proteolysis of the section (trypsin digestion) to enhance immunoreactivity, probably reflecting a hidden or sterically hindered epitope. However, the fact that two different antisera against HO-1 both gave similar immunostaining patterns greatly increases the likelihood that we are specifically recognizing HO-1 or an HO-1-like protein rather than a cross-reacting protein epitope.

To confirm the specificity of HO-1 binding, several control experiments were performed in parallel. No specific staining was seen with preimmune rabbit antisera or with rabbit antibodies against irrelevant epitopes (data not shown). Furthermore, adsorption with purified HO-1 protein completely abolished immunostaining of the HO-1 antiserum (Figure 1, D), whereas no effects were observed by adsorption of anti-τ serum with HO-1 protein (data not shown).

Tissue from young controls exhibited no HO-1 immunoreactive structures, whereas hippocamus from aged-matched control showed only occasional HO-1 immunoreactivity (Figure 1, B) in association with the few pathological structures (neurofibrillary tangles, senile plaque neurites, and neuropil threads) that are invariably found in aged brain. In addition, the neurofibrillary tangles characteristic of subacute sclerosis panencephalitis and progressive supranuclear palsy contained HO-1-like epitopes. In concordance with our findings in brain tissue from Alzheimer's disease and neurological controls, little or no HO-1 im-

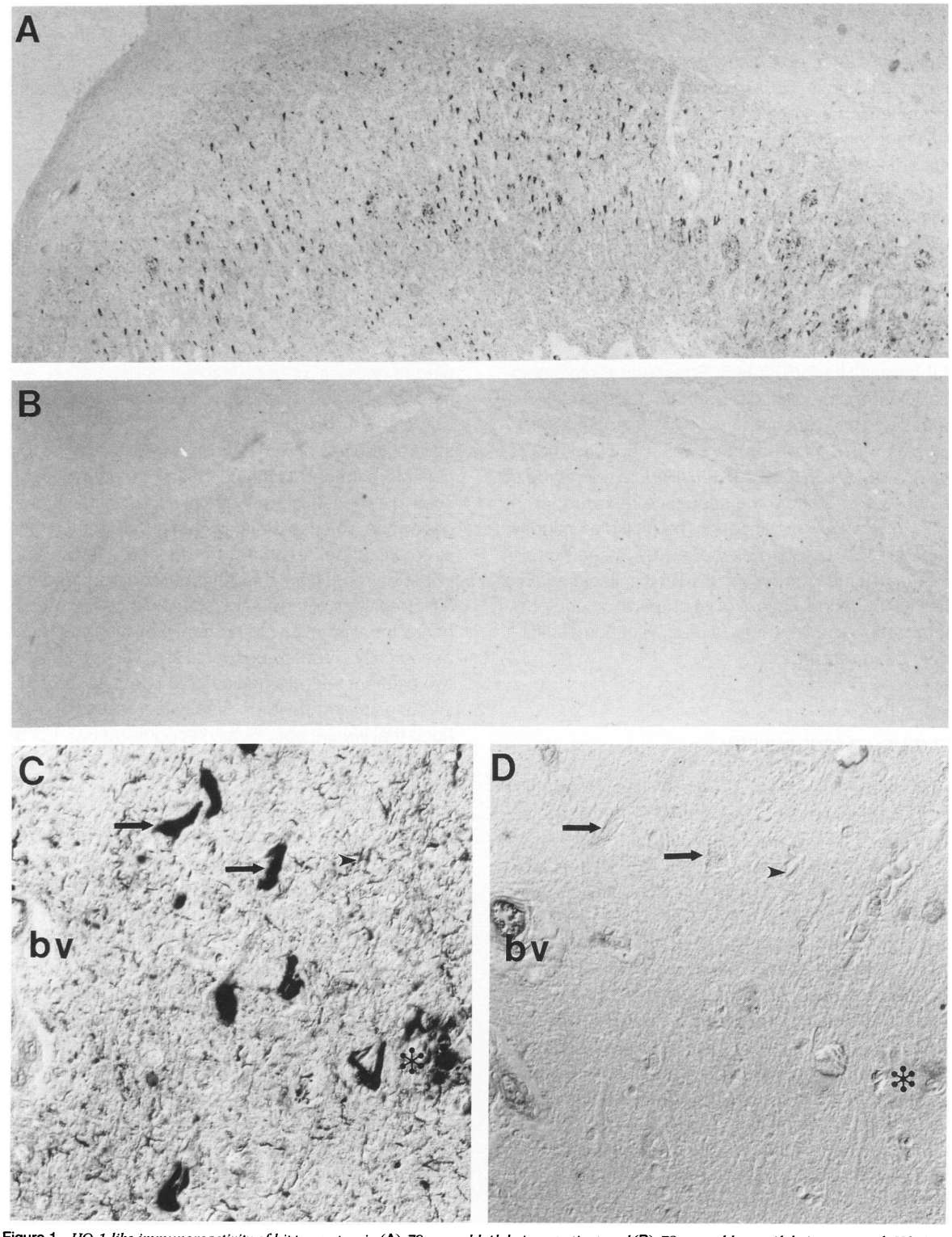

Figure 1. *HO-1-like immunoreactivity of hippocampus in* (A) *79-year-old Alzheimer patient and* (B) *78-year-old non-Alzheimer control. HO-1 immunoreactivity of* (C) *neurofibrillary tangles* (arrows), *senile plaque neurites* (*), *and neuropil threads* (arrowhead) *was* (D) *completely adsorbed in an adjacent section by preincubation of anti-HO-1 with purified HO-1 protein. bv represents landmark blood vessels in adjacent tissue section* (A,B ×40, C,D ×300).

munoreactivity was seen in neurons lacking neurofibrillary tangles. Again, the location and quantitation of the lesions was verified by anti-τ immunostaining of adjacent tissue sections.

Discussion

In this study, we immunocytochemically demonstrate the association of HO-1 with Alzheimer's disease pathology and the neurofibrillary tangles of progressive supranuclear palsy and subacute sclerosing panencephalitis, which lack amyloid β-protein. In Alzheimer's disease pronounced HO-1 immunoreactivity is seen localized to neurofibrillary tangles, senile plaque neurites, neuropil threads (ie, the neurofibrillary pathology), and granulovacuolar degeneration. In adjacent sections, HO-1-labeled structures show near total overlap with τ-positive structures suggesting that almost every pathological lesion contains increased HO-1. Control brains show no HO-1 immunoreactivity staining in young controls and sporadic lesion-related labeling in aged-matched controls.

The close overlap of HO-1 immunoreactive profiles seen in Alzheimer's disease and other conditions characterized by neurofibrillary tangles with other antigenic markers such as τ led us to conduct an amino acid homology search for possible cross-reactive proteins. Using a protein sequence data base (Swiss & PIR and Translated Release 78) there are no homologous proteins above a threshold of 50% using a K-Tuple of 3. Furthermore, direct sequence homology analysis using the Clustal method with a PAM250 residue weight table shows no significant regions of homology between HO-1 and τ, ubiquitin, hsp70, MAP2, tubulin, or neurofilament protein (heavy, medium, and light subunits).

There are several possible explanations why HO-1 protein levels are greatly increased in neurofibrillary tangles in Alzheimer's disease and other neurodegenerative diseases. First, HO-1 is induced during oxidative stress[27–29] to generate bilirubin, a potent antioxidant. Free radicals produced during oxidative stress are speculated to be pathologically important in Alzheimer's disease and other neurodegenerative diseases.[30] Indeed, we recently demonstrated that neurofibrillary tangles and senile plaques are associated with posttranslational modifications typical of nonenzymatic advanced Maillard reaction end products.[31,32] Such Maillard modifications are initiated and potentiated during oxidative stress[33] and would generate cross-linked aggregates of insoluble protein characteristic of senile plaques and neurofibrillary tangles.[34–36] Interestingly, the staining patterns of advanced Maillard end products and HO-1 have considerable overlap, suggesting that these epitopes might be pathology related, ie, by oxidative stress-type mechanisms.

Second, oxidative complications would also be important in the action of apolipoprotein E (ApoE), where individuals that are heterozygous or homozygous for the ApoE4 isoform are at greater risk of developing Alzheimer's disease.[37] ApoE forms sodium dodecyl sulfate-resistant complexes with amyloid-β protein, the main proteinacious component of senile plaques, and the formation of ApoE-amyloid-β protein complex is increased in oxygenated buffer and completely abolished under reducing conditions.[38] Interestingly, the oxidized ApoE4 gene product forms complexes with amyloid-β at a rate substantially higher than in similar experiments with the ApoE3 gene product. Therefore, the oxidation of ApoE alone or bound with amyloid-β protein might affect receptor affinity and/or other catabolic interaction in an analogous mechanism to the oxidation of low density lipoprotein in diabetic renal disease.[39]

Many heat shock proteins including HO-1 are regulated by the binding of a heat shock transcription factor to the heat shock elements found upstream of the TATA box in heat shock promoters.[40] The localization of several heat shock proteins in association with Alzheimer pathology and intracellular inclusions in other neurodegenerative diseases (including progressive supranuclear palsy and subacute sclerosing panencephalitis) suggests that there may be a generalized mechanism of induction of these heat shock proteins. The presence of HO-1 within neurofibrillary tangle-containing neurons but relative absence in adjacent 'normal-looking' neurons suggests that induction of HO-1 is mediated by intraneuronal stress mechanisms that are coincident with the formation or presence of cytoskeletal pathology. Moreover, progressive supranuclear palsy and subacute sclerosing panencephalitis, unlike Alzheimer's disease, do not contain amyloid β-protein deposits, indicating that amyloid deposition is not a prerequisite for HO-1 induction. However, in Alzheimer's disease the presence of amyloid β-protein might exacerbate neuronal atrophy and neurofibrillary degeneration.

Glutathione depletion induces HO-1 mRNA expression and protein level.[14] However, in Alzheimer's disease, glutathione levels are stable or increased compared with normal levels and glutathione peroxidase levels are unchanged.[41,42] Therefore, the increased expression of HO-1 protein in Alzheimer's disease is probably not the result of decreased glutathione levels.

Alzheimer's disease, progressive supranuclear palsy, and subacute sclerosing panencephalitis are all neurodegenerative conditions characterized by neuronal death and the accumulation of intraneuronal filaments. The mechanism of cell death in these conditions is unknown. However, as mentioned earlier, carbon monoxide, a reaction product of the enzymatic conversion of heme to biliverdin by HO-1,[1–2] is a putitive neurotransmitter.[3] Therefore, it is tempting to speculate that, like glutamate excitation-induced neuronal atrophy,[43] an increase in carbon monoxide neurotransmitter could lead to excitotoxicity and neuronal death. Such a scenario would explain the apparent progression of neuronal degeneration and lesion accumulation along corticocortical connections[44] in Alzheimer's disease.

In conclusion, the presence of HO-1 immunoreactivity in association with the neurofibrillary pathology of Alzheimer's disease and other neurodegenerative diseases provides evidence that oxidative stress and free radicals may play a role in disease etiology or pathogenesis and suggests a possible role of carbon monoxide in the pathological processes.

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