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Evidence for a Novel Heme-Binding Protein, HasAh, in Alzheimer Disease

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ABSTRACT

Multiple lines of evidence indicate that oxidative stress is an integral component of the pathogenesis of Alzheimer disease (AD). The precipitating cause of such oxidative stress may be misregulated iron homeostasis because there are profound alterations in heme oxygenase-1 (HO-1), redox-active iron, and iron regulatory proteins. In this regard, HasA, a recently characterized bacterial protein involved in heme acquisition and iron metabolism, may also be important in the generation of reactive oxygen species (ROS) given its ability to bind heme and render iron available for free radical generation through the Fenton reaction. To study further the role of heme binding and iron metabolism in AD, we show an abnormal localization of anti-HasA to the neurofibrillary pathology of AD, but not in normal-appearing neurons in the brains of cases of AD or in age-matched controls. These results suggest the increased presence in AD of a HasA homologue or protein sharing a common epitope with HasA, which we term HasAh. We conclude that heme binding of HasAh is a potential source of free soluble iron and therefore toxic free radicals in AD and in aging. This furthers the evidence that redox-active iron and subsequent Fenton reaction generating reactive oxygen are critical factors in the pathogenesis of AD. Antiox. Redox Signal. 2, 000–000.

INTRODUCTION

THERE IS GROWING EVIDENCE that free radical damage and oxidative stress play a pivotal role in the pathogenesis of Alzheimer disease (AD). However, whereas oxidative damage and antioxidant response are well characterized in AD (Smith *et al.*, 1991; Pappolla *et al.*, 1992; Smith *et al.*, 1994, 1995, 1996a,b), the source(s) of damaging reactive oxygen species (ROS) initiating such damage are still being investigated. As noted previously (Smith *et al.*, 1997), a known and potent source of free radicals arises from the Fenton reaction, where iron(II) is stoichiometrically oxidized by hydrogen peroxide (H_2O_2) to iron(III), producing a hydroxyl radical. Within cells, free radical production from iron(II) is catalytic because of redox cycling of iron(III) back to iron(II) at the expense of endogenous reducing species.

In AD, there is significant evidence for disrupted iron homeostasis (Thompson *et al.*, 1988; Connor *et al.*, 1992a,b; Good *et al.*, 1992; Smith *et al.*, 1997, 1998), suggesting iron as a source of free radicals. In particular, redox active iron is associated with neurofibrillary tangles and senile plaques of AD, and is a likely contributor to the pervasive oxidative damage found in AD. However, the source of redox-active iron itself remains to be elucidated. Nonetheless, we

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previously demonstrated abnormalities in the localization of iron regulatory proteins, which regulate iron homeostasis by enabling the tight coordination between the cytoplasmic concentration of free iron, cellular iron uptake, and the synthesis of ferritin and heme (Smith *et al.*, 1998). Furthermore, we found a parallel distribution of redox-active iron and heme oxygenase-1 (HO-1) in AD (Smith *et al.*, 1994), the latter being an enzyme responsible for the conversion of heme into anti-oxidant tetrapyrroles and free iron.

Because we suspect that heme is a major source of redox-active iron in the brain under pathological conditions, in this study, to investigate further the role of heme and heme iron as a potential source of free soluble iron in AD, we examined AD brains for the presence of a homologue to the heme-binding protein HasA (Izadi *et al.*, 1997).

HasA is a bacterial heme-binding protein that is present in several species (*Serratia marcescens, Pseudomonas aeruginosa,* and *Yersinia pestis*) (Letoffe *et al.*, 1998), although a HasA homologue has not yet been found in eukaryotic DNA sequence libraries. In addition, the crystal structure of HasA has been published recently (Arnoux *et al.*, 1999) and does not show structural similarities to other heme-binding proteins. Given the important role of iron in AD pathogenesis, we were interested in whether a HasA homologue (HasAh) was present in AD brains as a possible contributor to high levels of redox-active iron in AD.

MATERIALS AND METHODS

Tissue

Hippocampal and cortical tissue from 6 cases of AD (ages 69–84) and 8 controls (ages 31–82 years) were fixed in methacarn (methanol/chloroform/acetic acid; 60:30:10) at 4°C overnight.

Antibodies

Rabbit polyclonal HasA antiserum (Izadi *et al.*, 1997) at 1:50 dilution was used for this study. Antibodies to τ protein (Perry *et al.*, 1991) and ubiquitin (Manetto *et al.*, 1988) were used to confirm the identity and location of neurofibrillary pathology. For adsorption studies, purified HasA (1 mg/ml) with 10% hemoprotein and purified HasA (1 mg/ml) saturated with hemin were used.

Iron(II/III) histochemical detection

After deparaffinization in xylene and rehydration through graded ethanol, sections were incubated for 15 hr in 7% potassium ferrocyanide [for iron(III) detection] or 7% potassium ferricyanide [for iron(II) detection] in aqueous hydrochloric acid (3%) and subsequently incubated in 0.75 mg/ml 3,3′-diaminobenzidine and 0.015% H_2O_2 for 5–10 min.

Immunocytochemistry

Following fixation, tissue was dehydrated through ascending ethanol, and embedded in paraffin; 6-μm sections were placed on silane-coated slides. Tissue sections were immunostained by the peroxidase-antiperoxidase method with 3,3′-diaminobenzidine as cosubstrate as previously described (Sternberger, 1986). Pretreatment of tissue sections with 70% formic acid for 5 min increased the immunolabeling of the HasA antiserum. Adjacent sections were immunostained with antiserum to ubiquitin (Manetto *et al.*, 1988) or τ (Perry *et al.*, 1991). To verify the specificity of our findings, the primary antisera were absorbed with 0.70 μg/μl of apo- or holoprotein overnight at 4°C prior to application to the section followed by the peroxidase-antiperoxidase method. Other control experiments included omission of primary antisera and the use of preimmune antisera. Additionally, we found no cross-reactivity between HasA and human hemoglobin (native and denatured) with the polyclonal antisera to HasA.

RESULTS

Immunocytochemistry

AD Cases: Sections of medial temporal lobe, including the hippocampal formation, showed strong HasA-immunoreactivity localized predominantly to neurofibrillary tangles (Fig. 1A) in 5 out of 6 AD cases. Not surprisingly, this pattern of staining showed significant overlap with the distribution of redox-active iron, the latter of which was demonstrated by us previ-

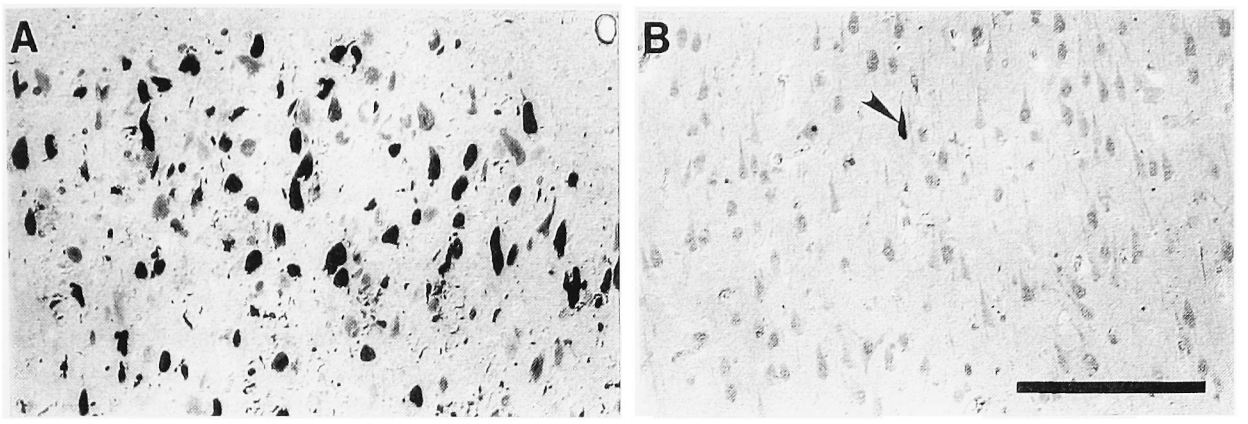

FIG. 1. HasAh immunoreactivity is markedly increased in the brain of an AD case (A) in comparison to an age-matched control (B). Importantly, while there is significant co-localization of HasAh with neurofibrillary pathology in AD (A), even sparse pathology in control cases (arrowhead) contain HasAh. Scale bar = 200 μm.

ously (Smith *et al.*, 1997) (Figure 2A–D). Immunostaining of neuropil threads and dystrophic neurites was also present, although these were stained slightly less than with τ immunohistochemistry performed on adjacent sections. Immunostaining of amyloid present in neuritic plaques was minimal in all cases whereas blood vessels involved by amyloid angiopathy show nonspecific background staining. Normal-appearing neurons were essen-

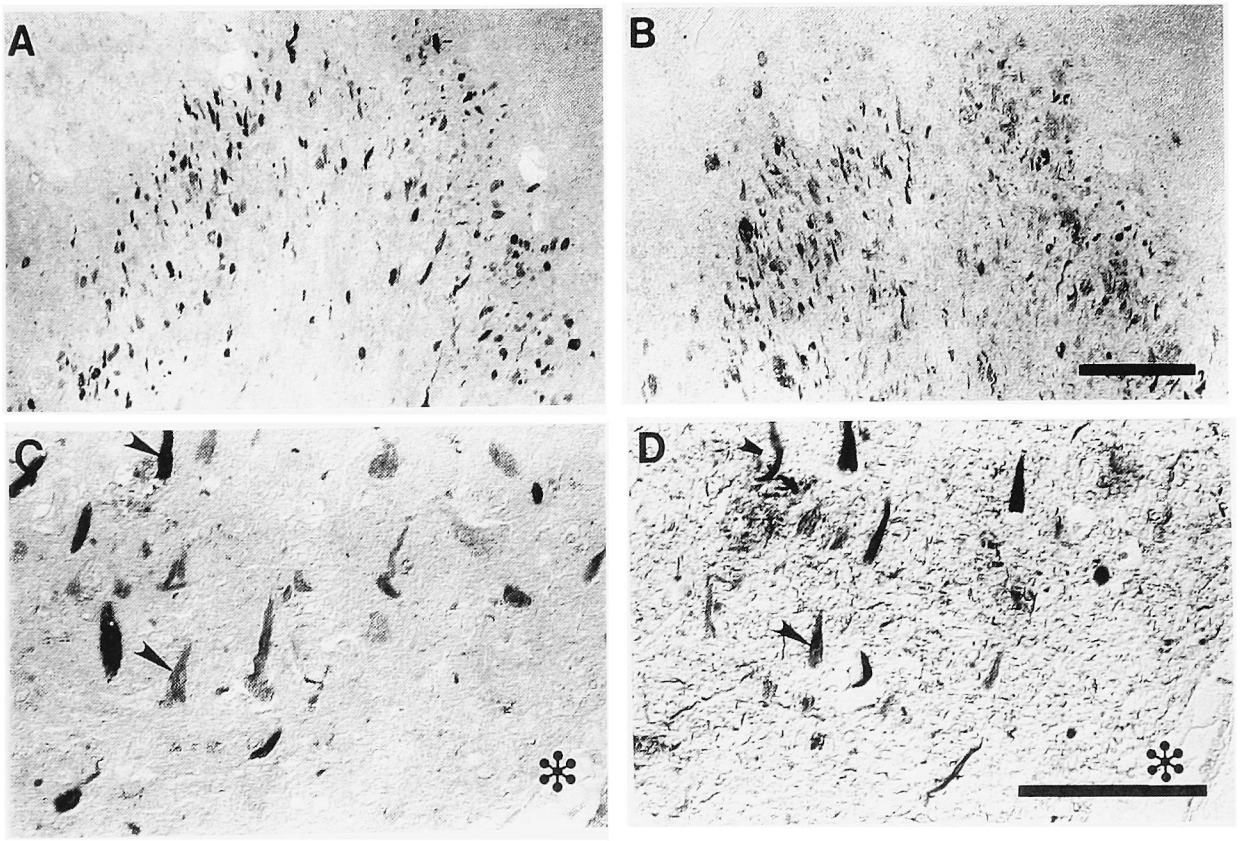

FIG. 2. The significance of HasAh protein (A,C) in the pathogenesis of AD is demonstrated by an almost complete overlap with redox-active iron (B,D), a potent source of free radicals. Scale bars = 200 μm (A,B) and 100 μm (C,D).

tially unstained, as were glial cells in both gray matter and subcortical white matter.

Control Cases: Hippocampal sections in 8 age-matched controls showed no immunostaining of normal-appearing neurons or glia (Fig. 1B), and no significant vascular immunoreactivity. In 5 of the age-matched control cases, some neurofibrillary pathology was identified by both anti-HasA and anti-τ immunohistochemistry; however, in 3 of these cases, τ immunoreactivity was more widespread than the HasA staining, and in 2 cases, the amount of HasA and τ staining were approximately the same.

Adsorption

Sections of hippocampus from AD cases immunostained with an antibody to HasA, which had been adsorbed with recombinant Apo-HasA protein, showed diminution in immunoreactivity and even greater reduction if recombinant Holo-HasA protein containing heme was used as adsorbent (Fig. 3A–C). This differential adsorption pattern not only indicates the specificity of the antibody but also suggests that the HasA in AD represents the holo form of the protein. Further attesting to the specificity of our findings, a preimmune sera only resulted in background labeling. Taken together, our results are consistent with the presence of HasA or HasA-like protein (HasAh) bound to iron-containing heme, localized predominantly to neurofibrillary pathology of AD.

DISCUSSION

In this study, we demonstrate the presence of the heme-binding protein, HasAh, in association with the lesions of AD. That the distribution of HasAh parallels that of both redox-active iron and HO-1 furthers the notion that dysregulation of iron homeostasis is critical to the pathogenesis of AD. Furthermore, the colocalization of oxidative adducts with HasAh together with the known prooxidant ability of heme (or iron) indirectly implicates free radical damage via the Fenton reaction as an integral component of AD.

HasA is a recently characterized 19-kDa protein that can bind free heme and acquire it from hemoglobin (Izadi *et al.*, 1997). It is necessary for the utilization of iron by bacteria and it acts as a heme carrier. HasA belongs to a family of secreted proteins lacking an amino-terminal signal peptide. It has a carboxy-terminal targeting sequence and is secreted by a specific ATP binding cassette (ABC) transporter, composed of three envelope proteins: an ABC protein, a membrane fusion protein located in the inner membrane, and an outer membrane protein. HasA does not present sequence homology with other known proteins, including other hemoproteins. The only sequence similarity between HasA and other proteins was found with proteins secreted via an ABC transporter. Although a eukaryotic DNA encoding protein homologous to HasA had not been reported, the HasA immunoreactivity demonstrated in this study suggests that a HasA homologue, which

FIG. 3. HasAh immunoreactivity in an AD case (A) is reduced by adsorption of the primary antisera with apo- (B) or holo-(C) HasA protein. Notably, labeling is markedly reduced using the holo-form of HasA in comparison to the apo-form, indicating that in AD brain the predominant form of HasA is likely in the holo form. Scale bar = 100 μm.

we term HasAh, is likely encoded in the human genome. Analysis of human cDNA expresion libraries in recombinant *Escherichia coli* with anti-HasA antibodies will help in answering such questions.

The presence of HasAh in AD lesions is consistent with a number of features of iron metabolism in AD that we have demonstrated previously. These include: (i) up-regulation of HO-1 (Smith *et al.*, 1994); (ii) abnormal localization of iron regulatory protein-2 (Smith *et al.*, 1998); and (iii) the presence of redox-active iron(II) in the pathological lesions of AD (Smith *et al.*, 1997; Castellani *et al.*, 1999). Because HasA is a secreted protein under normal conditions, we are unable to establish whether the increased but discrete protein distribution in AD to the lesions either represents an upregulation by diseased, tangle-bearing neurons, or, alternatively, HasAh could be secreted by healthy cells and sequestered by the lesion. In either case, it is difficult to implicate HasAh as a early abnormality in the pathogenesis of AD on the basis of its localization to end-stage lesions. Indeed, in those age-matched controls that contained incidental neurofibrillary tangles, HasAh staining tended to identify fewer pathological neurons than did τ, suggesting that HasAh upregulation follows the basic alterations that lead to neurofibrillary tangle formation. Nevertheless, the presence of HasAh in pathological structures in AD suggests that HasAh-heme binding may represent an important positive feedback mechanism for the generation of free radicals and may accelerate progression of disease.

Because HasA normally functions to acquire heme from hemoglobin with high-affinity binding (Izada *et al.*, 1997), heme itself is likely present along with HasAh in the neurofibrillary pathology of AD. Although we do not provide direct evidence for increased heme in AD brains, the results of our adsorption experiments with heme protein, along with our HasAh results and the co-distribution of HasAh and iron(II), suggest increased heme content in pathological lesions as a source of iron and therefore free radicals. A source of heme, in turn, requires additional studies; however, on the basis of parallel studies, mitochondria are a likely candidate. Such a notion is supported by (i) the known high heme content in mitochondria; (ii) studies showing increased mitochondrial DNA, both mutant and wild type, in aged brains; and (iii) increased mitochondrial 4977 base pair deletions in brain and skeletal muscle associated with oxidative stress and aging (Corral-Debrinski *et al.*, 1992). To examine this aspect further, we are now determining whether there is direct evidence for mitochondrial abnormalities, which, if present, would further the argument that mitochondrial-derived heme is a major source of iron (II)-derived free radicals.

In summary, in this study, we demonstrate the abnormal localization of a HasA-like heme binding protein, HasAh, in the neurofibrillary pathology of AD brains, as well as in incidental neurofibrillary tangles of age-matched controls. These results implicate heme, likely of mitochondrial origin, as a potential source of redox-active iron and therefore toxic free-radical generation in AD and in aging. By implication, this then furthers the evidence that redox-active iron and the Fenton reaction are critical factors in AD pathogenesis, and supports the role of anti-oxidant and chelation therapy for potentially slowing disease progression.

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ABBREVIATIONS

ABC, ATP binding cassette; AD, Alzheimer disease; HO-1, heme oxygenase-1; H_2O_2, hydrogen peroxide; ROS, reactive oxygen species.

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