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Iron accumulation in Alzheimer disease is a source of redox-generated free radicals

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ABSTRACT **Damage from free radicals has been demonstrated in susceptible neuronal populations in cases of Alzheimer disease. In this study, we investigated whether iron, a potent source of the highly reactive hydroxyl radical that is generated by the Fenton reaction with H_2O_2, might contribute to the source of radicals in Alzheimer disease. We found, using a modified histochemical technique that relies on the formation of mixed valence iron complexes, that redox-active iron is associated with the senile plaques and neurofibrillary tangles—the pathological hallmark lesions of this disease. This lesion-associated iron is able to participate in *in situ* oxidation and readily catalyzes an H_2O_2-dependent oxidation. Furthermore, removal of iron was completely effected using deferoxamine, after which iron could be rebound to the lesions. Characterization of the iron-binding site suggests that binding is dependent on available histidine residues and on protein conformation. Taken together, these findings indicate that iron accumulation could be an important contributor toward the oxidative damage of Alzheimer disease.**

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 (1–5), the source(s) of damaging reactive oxygen species initiating such damage is yet to be established. A known and potent source of free radicals arises from the Fenton reaction, where iron(II) is stoichiometrically oxidized by H_2O_2 to iron(III), producing a hydroxyl radical (OH·). 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.

While there is evidence for altered iron metabolism in AD, including alterations in iron, transferrin, and ferritin (6–9), the extent of iron accumulation or whether such iron participates in redox chemistry is not established. We examined the distribution of iron in the brain of cases of AD employing a modification of a well-established histochemical stain (10), based on the Prussian blue reaction (11). This method involves binding of iron(II) cyanide (ferrocyanide) to iron(III) in tissue to give a brilliant blue mixed-valence iron(II/III) complex, which can be observed directly or that can be enhanced, based on the ability of the mixed-valence complex to catalyze a peroxidase-like H_2O_2-dependent oxidation of 3,3′-diaminobenzidine (12).

MATERIALS AND METHODS

Tissue Sections. Hippocampal tissue from 10 cases of AD (ages 70–94, average age 81.4) and from 5 controls (ages 31–82, average age 55) with similar postmortem intervals were fixed in methacarn (methanol:chloroform:acetic acid, 60:30:10) at 4°C for 15 hr. Following fixation, tissue was placed in 50% ethanol, dehydrated in ascending ethanol, and embedded in paraffin. Sections (6 μm) were mounted onto silane-coated slides (Sigma). Alternatively, fresh unfixed tissue was frozen at autopsy in isopentane chilled with liquid nitrogen and subsequently sectioned (10 μm) with a cryostat.

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.

Three key modifications of the established histological method (10, 11) greatly increased the detection sensitivity, namely: (*i*) increasing the concentrations of potassium ferrocyanide (7%) and hydrochloric acid (3%); (*ii*) lengthening the incubation time and/or temperature (1 hr at 37°C or 15 hr at room temperature); and (*iii*) use of methacarn versus formalin-fixed tissue. The last modification is perhaps the most critical to iron detection in human tissue where formaldehyde-based fixation for extended periods is routine. Indeed, we found that even a brief postfixation with formaldehyde (3.7%, 5–60 min at room temperature) reduced labeling in a time-dependent manner (results not shown). The results with methacarn fixation are similar to those found in sections made from frozen blocks, which were neither fixed nor embedded, suggesting that the iron localization is not a result of tissue fixation or embedding (data not shown).

***In Situ* Oxidation.** Redox-active iron was directly demonstrated by incubation of the tissue sections with 3% H_2O_2 and 0.75 mg/ml 3,3′-diaminobenzidine.

Iron Chelation/Binding. Iron was completely stripped from neurofibrillary tangles by a 20 min exposure to 0.1 M deferoxamine at room temperature. However, a 15 hr incubation was required for complete removal of iron bound to senile plaques. Use of 0.1 M EDTA for 15 hr was only partly effective in removing iron. Rebinding was accomplished by incubation with 0.01 mM iron(III) citrate and 0.01 mM iron(II) chloride at room temperature for 3 hr.

Protein Modifications. The following modifications were assessed: heparan removal using heparinase/heparatinase [1 unit of each per ml (Sigma) in 10 mM Tris/20 mM $CaCl_2$, pH 8.0, for 15 hr at room temperature; ref. 13]; chondroitin removal with chondroitinase ABC [1 unit/ml (ICN) in 10 mM Tris/20 mM $CaCl_2$, pH 8.0, for 15 hr at room temperature; ref. 14]; phosphate removal with alkaline phosphatase [400 μg/ml (Sigma) in 0.01 M phenylmethylsulfonyl fluoride/0.1 M Tris, pH 8.0, overnight at room temperature; ref. 13]; protein denaturation [1,1,1,3,3,3-hexafluoro-2-propanol (Fluka) for 30 min at room temperature; ref. 15]; blockage of histidine

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Abbreviation: AD, Alzheimer disease.

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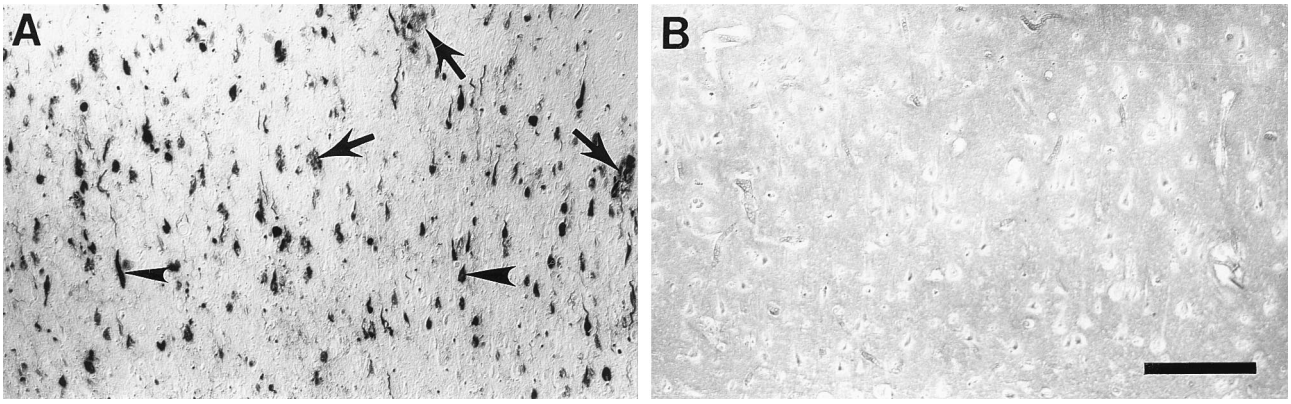

FIG. 1. Histochemical detection of iron in AD (*A*) compared with control cases (*B*) show striking association of iron with neurofibrillary tangles (arrowheads) and senile plaques (arrows) characteristic of the AD brain. (Scale bar = 200 μm.)

residues (5 mM diethyl pyrocarbonate in 0.1 M PBS, pH 7.2, for 30 min at 4°C; ref. 16); reversal of the histidine modification [0.1 M hydroxylamine hydrochloride (Aldrich) in 0.1 M PBS, pH 7.2, for 30 min at 4°C; ref. 17.

RESULTS AND DISCUSSION

Striking accumulations of iron(III), localized with iron(II) cyanide (ferrocyanide), were closely associated with senile plaques, neurofibrillary tangles, and neuropil threads (i.e., the pathological lesions) in cases of AD (Fig. 1*A*), whereas no specific structure was stained in the brains of control cases (Fig. 1*B*). It is of interest that iron was specifically localized to lesions of AD and not the glial cells surrounding senile plaques, which contain abundant iron binding proteins (6, 18). This aspect suggests the lesion-associated iron is distinct from that sequestered by normal storage proteins. Evidence for the presence also of iron(II) was that (*i*) treatment with H_2O_2, i.e., converting iron(II) to iron(III), increased labeling with potassium ferrocyanide and (*ii*) iron(III) cyanide (ferricyanide) labeled the same pathological structures. Theoretically, it should be possible to identify iron(II) and iron(III) independently in tissue by staining with ferricyanide and ferrocyanide, respectively, to form the same mixed valence iron(II)/(III) complex. However, this assumes that the bound iron is both accessible and in a redox immutable form during tissue preparation and histochemical analysis. This concern is highlighted by studies that show that at least some iron(III) proteins can be histochemically localized using iron(III) cyanide (19), implying that endogenous reducing species are recruited to form the mixed valence iron(II)/(III) complex in these cases. Therefore, although differential staining by the iron(II) and iron(III) cyanide complexes may actually reflect localization of the two iron redox states, such interpretation must recognize the potential for endogenous redox reactions.

Two findings showed that the iron we are detecting is not an artifact of the iron-containing reagents employed in the histochemical technique. First, we found no labeling of tissue exposed to ferrocyanide following prior removal (chelation) of iron with deferoxamine. Significantly, following removal of tissue iron by chelation, iron could be reasssociated with the neurofibrillary tangles and senile plaques, i.e., a distribution identical to that found before removal (Fig. 2), but only by using *exchangeable* sources of iron [we used a mixture of iron(III) citrate and iron(II) chloride]. Second, endogenous redox-active iron was *directly* demonstrated in the pathological lesion, by *in situ* catalysis of H_2O_2-mediated oxidation of 3,3′-diaminobenzidine. Using this *in situ* oxidation paradigm, we found labeling of all the same structures visualized by using the histochemical techniques. That this observation reflects tissue-bound iron is indicated by the abolishment of 3,3′-diaminobenzidine oxidation with prior treatment with deferoxamine (M.A.S., L.M.S., and G.P., unpublished data). This result, along with our findings showing an increase in potassium ferrocyanide-mediated labeling following pretreatment with 3% H_2O_2, indicated that at least some of the tissue-bound iron is available for redox chemistry.

To examine which elements of senile plaques and neurofibrillary tangles were critical to iron binding, we chemically modified specific groups and evaluated their effect on iron re-association to the lesions (Fig. 2; Table 1). Enzymatic removal of the highly charged heparan or chondroitin sulfate proteoglycans, as well as phosphate groups, had no effect on iron rebinding, whereas diethyl pyrocarbonate modification of histidine residues, an amino acid often involved in metal coordination, completely abolished the ability of the lesions to

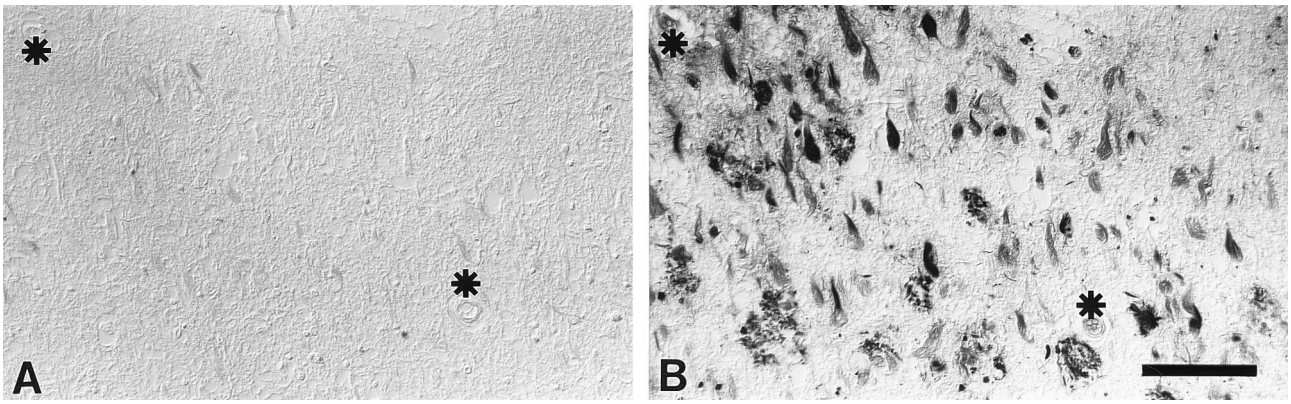

FIG. 2. Lesion-associated iron (see Fig. 1*A*) could be completely stripped with deferoxamine (*A*) but readily rebound to the same sites following incubation with iron(III) citrate and iron(II) chloride (*B*). * indicates landmark blood vessel in adjacent section. (Scale bar = 100 μm.)

Table 1. Chemical modification of residues that might bind iron in neurofibrillary tangles and senile plaques show that histidine residues and protein conformation are essential to iron binding

Modification	Treatment	Effect on iron binding
Heparan removal	Heparinase/heparitinase	None
Chondroitin removal	Chondroitinase ABC	None
Phosphate removal	Alkaline phosphatase	None
Histidine blockage	Diethyl pyrocarbonate	Blockage*
Protein conformation	Hexafluoroisopropanol	Blockage

*Importantly, reversal of the histidine modification completely restored iron binding potential to the lesions.

associate iron. Specificity of the diethyl pyrocarbonate treatment was confirmed by noting that deprotection of the modified histidine with hydroxylamine restored iron-rebinding capability (results not shown). Additionally, iron rebinding was sensitive to protein conformation, since treatment with the conformation-altering solvent hexafluoroisopropanol prevents association of iron (Table 1). Whether the binding of iron is to specific iron-binding proteins reportedly found in association with senile plaques and neurofibrillary tangles (7, 20), or is through adventitious binding to the abnormal protein constituents of these lesions, remains to be determined. In favor of the latter, the chelation and binding characteristics, as well as the ability to participate in redox reactivity, strongly indicate that the lesion-associated iron is not bound to normal iron binding proteins. Possible proteins shared between neurofibrillary tangles and senile plaques and with demonstrated metal binding sites include amyloid-β (21) and apolipoprotein E (22).

In the brain, the distribution of iron between (II) and (III) oxidation states would depend on the availability of reducing species, as well as on the nature of the protein coordination sites (23, 24). Our demonstration of redox activity of the lesion-bound iron suggests the potential for catalytic generation of free radicals at the expense of cellular reducing species. This hypothesis would be consistent with the restriction of oxidative damage to regions displaying neurofibrillary tangles and senile plaques in cases of AD (5, 25). While beyond the scope of the present report, it is of interest that ferricyanide, but not ferrocyanide, stained nuclei of AD and control brain. Moreover, while readily removable with deferoxamine and rebindable, this nuclei-associated iron was not able to catalyze the *in situ* H_2O_2-mediated oxidation of 3,3′-diaminobenzidine nor did ferrocyanide staining appear upon pre-exposure to H_2O_2, which would be expected to oxidize iron(II) to iron(III). The latter findings suggest that nuclei-associated iron, unlike the iron that is associated with the pathological lesions, is not available for redox reactivity.

While the source of iron that is associated with the lesions is unknown, it is of note that the pattern of redox-available iron in neurons is strikingly similar to the localization of heme oxygenase-1 (3), an enzyme responsible for the conversion of heme into antioxidant tetrapyrroles and free iron. Thus, in cases where there is chronic induction of heme oxygenase, such as in AD, enzyme inhibitors such as zinc protoporphyrin, along with iron chelation therapies, may slow iron accumulation.

In conclusion, our findings indicate alterations of iron homeostasis in AD, and this is supported by the recent demonstration of elevated serum levels of the iron binding protein p97 in patients with AD (26), indicating that such alterations might manifest in peripheral markers. Furthermore, the presence of redox-available iron in association with the pathological lesions shown here, coupled with the increasing number of reports implicating oxidative stress, strongly support the key role that oxidative damage plays in the pathogenesis of AD.

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