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Forum Review

DNA Oxidation in Alzheimer's Disease

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ABSTRACT

Oxidative damage to DNA may play an important role in aging and neurodegenerative diseases such as Alzheimer's disease (AD). Attack on DNA by reactive oxygen species, particularly hydroxyl radicals, can lead to strand breaks, DNA–DNA and DNA–protein cross-linking, sister chromatid exchange and translocation, and formation of at least 20 oxidized base adducts. Modification of DNA bases can lead to mutation and altered protein synthesis. In late-stage AD brain, several studies have shown an elevation of the base adducts 8 hydroxyguanine (8-OHG), 8-hydroxyadenine (8-OHA), 5-hydroxycytosine (5-OHC), and 5-hydroxyuracil, a chemical degradation product of cytosine. Several studies have shown a decline in repair of 8-OHG in AD. Most recently, our studies have shown elevated 8-OHG, 8-OHA, and 5,6-diamino-5-formamidopyrimidine in nuclear and mitochondrial DNA in mild cognitive impairment, the earliest detectable form of AD, suggesting that oxidative damage to DNA is an early event in AD and not a secondary phenomenon. *Antioxid. Redox Signal.* 8, 2039–2045.

CLINICAL MANIFESTATIONS OF ALZHEIMER'S DISEASE

ALZHEIMER'S DISEASE (AD) is the most common type of dementia in people over 65 years old, and affects 4.5 million persons in the United States (25). With the growth of the elderly population, it is predicted that 14 million individuals will be affected by the year 2050 (24). AD is characterized by an insidious decline in cognitive function from a previous higher level of function that results in impairment of social or occupational functions. The disease involves impairment in recent memory, language disturbances, visual processing dysfunction, praxis disturbances, and disturbance of executive function (abstract reasoning, concentration, and sequencing) (1). The National Institute of Neurological and Communicative Diseases and Stroke/Alzheimer's Disease and Related Disorders Association (NINCDS-ADRDA) criteria (40) for the clinical diagnosis of probable AD include (a) dementia established by clinical examination and documented by mental status tests, (b) deficits in two or more areas of cognition, (c) progressive worsening, (d) no disturbance in consciousness, (e) onset between 40 and 90, and (f) no systemic or other brain disease that could account for the progressive deficit. Progression of the disease leads to severe dementia with disorientation, profound memory impairment, and eventually to global cognitive deficits and immobility. Average length of life after diagnosis is 8.5 years (30).

AD often begins with amnestic mild cognitive impairment (MCI). The concept of MCI as a phase between normal aging and early dementia and AD has served as a strong stimulus for early detection. The criteria for amnestic MCI include (a) memory complaints, preferably corroborated by an informant, (b) objective memory impairment for age and education, (c) intact general cognitive function, (d) intact activities of daily living, and (e) the subject is not demented (52). Conversion from MCI to dementia occurs at a rate of 10–15% per year (52), although approximately 5% remain stable or revert back to normal (4, 13).

NEUROPATHOLOGICAL FINDINGS OF ALZHEIMER'S DISEASE

The major diagnostic pathological features of AD are the presence of neurofibrillary tangles (NFT) and beta amyloid

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(Aβ) plaques in the entorhinal cortex, hippocampus, amygdala, and association areas of frontal, temporal, parietal, and occipital cortex. NFT are cytoplasmic masses of paired helical filaments containing hyperphosphorylated tau. There are two major forms of Aβ plaques: neuritic plaques (NP) and diffuse plaques. NP are composed of extracellular deposits of insoluble Aβ surrounded by dystrophic neurites, reactive astrocytes, and activated microglia. Diffuse plaques are amorphous extracellular deposits of Aβ-reactive granular material that lack neurites. It appears that diffuse plaques precede NP and may be the precursors of NP. A recent study indicates there is a combination of soluble and oligomeric forms of assembled Aβ in AD (20), although the contribution of the different assembled forms of Aβ to neuropathological and neurochemical alterations in AD remains to be clarified. In addition, AD pathology includes variable focal neuron loss, synapse loss, astrocytosis, and deposition of Aβ in small blood vessels.

Braak and Braak (6) defined a hierarchical order of the involvement of neurofibrillary pathology that is useful in staging the severity of AD. They described three stages: stage I and II (transentorhinal stage), where NFT occur in the transentorhinal regions initially and eventually in the entorhinal cortex and to a lesser degree in the hippocampus. In stage III and IV (limbic stage), there are increased NFT in the entorhinal cortex, hippocampus, and amygdala. In stage V and VI (isocortical stage), there is progressive formation of NFT in neocortical association areas.

The two diagnostic criteria for the neuropathologic diagnosis of AD currently in use are the Consortium to Establish a Registry for Alzheimer's Disease (CERAD) criteria (45) and the National Institute on Aging–Reagan Institute (NIA–RI) criteria (2). The CERAD criteria rely on semiquantitation of NP density in middle frontal gyrus, superior and middle temporal gyri, and inferior parietal lobule. The density of NP is estimated as sparse, moderate, or frequent. Those ratios are then compared with the age of the patient to yield an age-related plaque score. The age-related plaque score is integrated with the clinical history of dementia to reach a diagnosis of possible, probable, or definite AD.

The NIA-RI criteria use the CERAD age-related plaque score and the Braak and Braak staging system to propose a probabilistic definition for the diagnosis of AD. The likelihood is low that AD is present if the CERAD score is infrequent and the Braak stage is I or II. There is moderate likelihood that the dementia is due to AD if the CERAD score is moderate and the Braak score is III or IV. When the CERAD score is frequent and the Braak stage is V or VI, there is a high likelihood that the dementia is due to AD. These criteria are more difficult to use when the CERAD score and the Braak stage are discordant. The criteria also are problematic when there is a lack of solid clinical data to define the presence of dementia. These criteria have not been validated in individuals 90–105 years old.

FREE RADICAL-MEDIATED OXIDATIVE DAMAGE

A free radical is defined as any atom or molecule with one or more unpaired electrons in its outer shell. Multiple radicals exist but the most common radicals are formed from the reduction of molecular oxygen to water and are referred to as reactive oxygen species (ROS). Oxidative stress is the condition that exists when free radicals and their products are in excess of antioxidant defense mechanisms. Oxidative stress leads to oxidative damage of molecules and the potential of decline or loss of their functions. There is increasing evidence that free radical-mediated damage to cellular function contributes to the aging process, cancer, and age-related neurodegenerative diseases. Considerable data indicate that oxidative damage may play a role in the pathogenesis of AD, Parkinson's disease, amyotrophic lateral sclerosis, and Huntington's disease (39).

Numerous studies have demonstrated increased lipid peroxidation, protein oxidation, DNA and RNA oxidation, and glycooxidation in multiple brain regions in AD (8, 37). Several immunohistochemical studies have shown that the oxidative damage is prominent in neuron cytoplasm (46, 49, 50). Many of these studies have used brain specimens from late stage AD patients and this does not allow us to know whether the oxidative damage is a late effect of the disease or if it occurs early in the disease process.

Most recently there have been multiple studies showing that lipid peroxidation, protein oxidation, and RNA and DNA oxidation occur in mild cognitive impairment, the phase between normal aging and early AD (9, 15, 32, 38, 57). This suggests that oxidative damage is an early event in AD.

DNA OXIDATION

Approximately 2% of oxygen consumed by cells during oxidative phosphorylation is converted into ROS (22). In 1956, Harman (23) suggested that these ROS lead to cell damage that could be the basis of cellular aging. Subsequently, Szilard (56) suggested that DNA was the primary target of ROS leading to cellular aging. Since that time, studies have shown that oxidative damage to DNA might be important in cancer (3, 59). Because of the high oxygen consumption rate by the brain, it is also possible that ROS may contribute to neuronal damage in aging and neurological disorders.

Oxidative attack of DNA by ROS, particularly hydroxyl radicals, can lead to strand breaks, DNA–DNA and DNA–protein cross linking; and in nuclear DNA (nDNA), sister chromatid exchange and translocation (11, 12). Additionally, ROS attack of DNA can lead to the production of more than 20 oxidized base adducts (10, 54), changes that are more subtle and require close analysis of DNA molecules to determine alterations (Fig. 1). DNA bases may also be modified by neurotoxic markers of lipid peroxidation including 4-hydroxynonenal and acrolein, leading to formation of bulky exocyclic adducts. Formation of modified DNA bases could result in alterations in replication of DNA or inappropriate base pairing producing mutations that could lead to altered protein synthesis. Mutations in nDNA increase with age (14, 29), and there is a suggestion that DNA repair may decline with aging.

Because it has the lowest oxidation potential of the four DNA bases, guanine is the most readily oxidized base

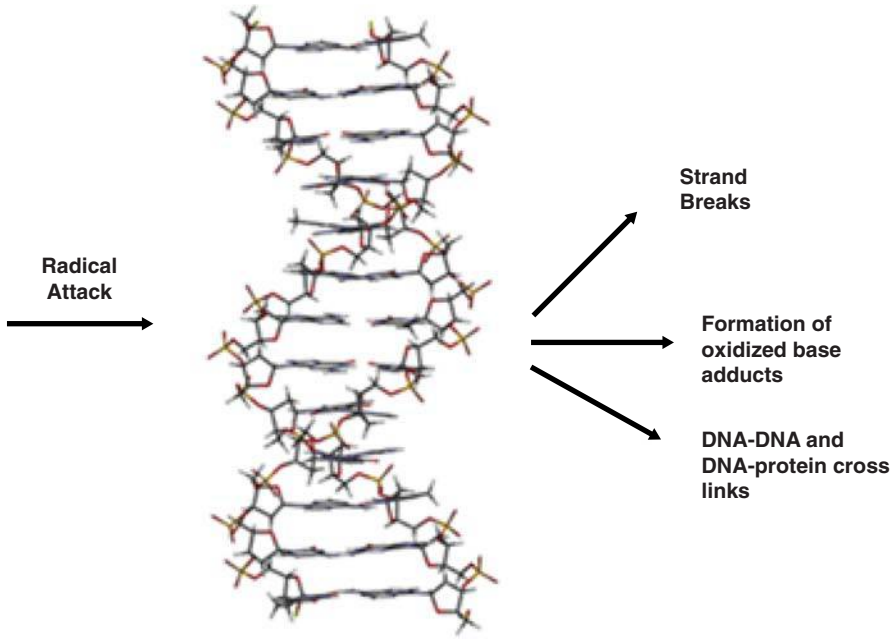

FIG. 1. Radical attack, particularly by hydroxyl radicals, can lead to strand breaks, DNA–DNA and DNA–protein crosslinks, and formation of more than 20 oxidized base adducts.

through free radical attack of C8 leading to the formation of 8-hydroxyguanine (8-OHG) under elevated oxygen tension and 2,6-diamino-4-hydroxy-5-formamidopyrimidine (fapy-guanine) under conditions of reduced oxygen tension (7, 54). [Fig. 2, adapted from Evans *et al*, (18)]. Because of the ease of oxidation and the presence of oxygen, 8-OHG is the most commonly studied marker of DNA oxidation and is measured as either 8-hydroxyguanosine using enzymatic digestion of DNA and high pressure liquid chromatography (HPLC) or 8-OHG using acid hydrolysis of DNA followed by derivatization and analysis using gas chromatography (GC). For continuity, 8-OHG will be used in this review to represent oxidized guanine regardless of the form (8-hydroxyguanosine or 8-hydroxyguanine) being measured. Previous studies show that 8-OHG is increased in aging brain (16, 21, 26, 41). In addition to 8-OHG and fapy-guanine, 8-hydroxyadenine (8-OHA), 4,6-diamino-5-formamidopyrimidine (fapy-adenine), and 5-hydroxycytosine (5-OHC) have also been observed to be altered in brain specimens in AD (19, 57, 58). Although present at lower absolute concentrations compared to 8-OHG, fapy-guanine is of importance in that it is formed from radical attack of C8 of guanine followed by opening of the ring under conditions of low oxygen tension. Similar adducts, 8-hydroxyadenine (8-OHA) and fapy-adenine, are formed during radical attack of C8 of adenine. Measurement of both adducts for a particular base provides information regarding the conditions under which the adducts were formed.

Mitochondrial DNA (mtDNA) oxidation, especially 8-OHG, is of a much greater magnitude than nDNA. Nuclear DNA gains some protection from histones and morphologically is not close to large oxidant generation. In contrast, mtDNA does not have protective histones, is in close proximity to oxidant generation, may not have a rich antioxidant system, and has relatively limited DNA repair capacity, which may account for some of its vulnerability to oxidation.

To measure DNA oxidation, particularly 8-OHG, several analytical approaches have been used including: a) ^{32}P or fluorescent postlabeling of nucleosides enzymatically digested from DNA, b) use of HPLC or GC coupled with mass spectrometry (MS) or capillary electrophoresis coupled with fluorescence or UV-Vis detection, and c) use of immunoassays (51). Of the three approaches, immunoassays have been primarily utilized in the analysis of 8-OHG in urine samples or other dilute biological solutions (51). For the analysis of oxidation of DNA derived from tissue specimens, HPLC with electrochemical detection has been one of the most widely used analytical techniques. Although this approach provides detection limits of ~20 fmol, there is potential interference by co-eluting compounds. A similar method employs capillary electrophoresis coupled with electrochemical, UV-Vis, or laser-induced fluorescence to quantify 8-OHG. Although capillary electrophoresis offers greater separation efficiency and likely reduces interferences from complex mixtures, the small volume used for analysis results in poorer sensitivity and detection limits compared with HPLC (51).

To reduce the potential for interference due to co-eluting species, more recent studies have employed HPLC or GC coupled with MS for unequivocal identification of base adducts. Additionally, these approaches allow the identification of multiple adducts in addition to 8-OHG. For HPLC analyses, DNA samples are enzymatically digested to nucleosides and subjected to solid phase extraction for sample clean-up. The resulting nucleosides are generally mixed with stable-labeled internal standards for liquid chromatography (LC)/MS and quantification. For GC/MS, DNA is hydrolyzed to individual bases using formic acid and derivatized to increase volatility. Quantification is based on comparison to stable labeled internal standards or an external calibration curve.

Because of the extensive sample preparation required for analysis of oxidized DNA bases, there has been concern re-

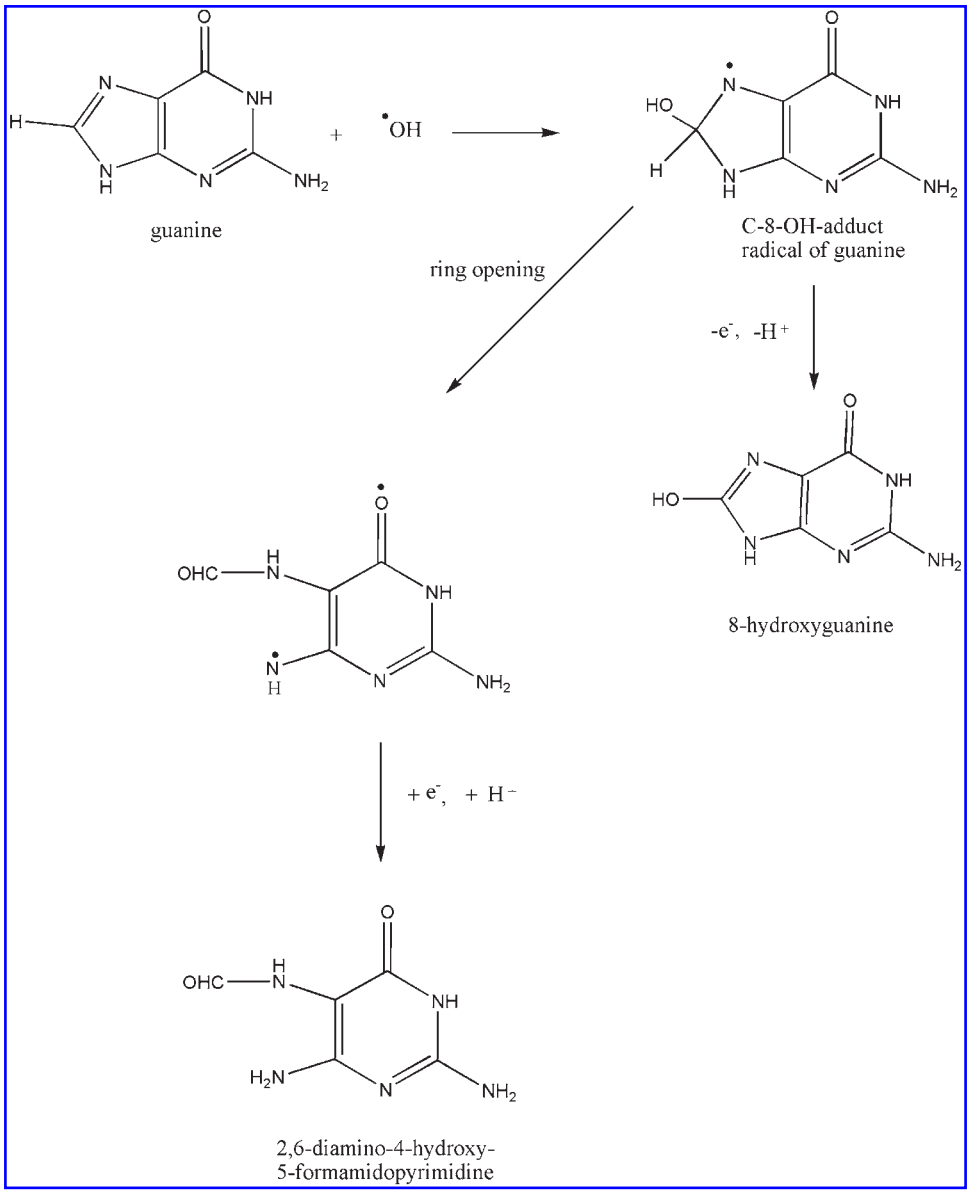

FIG. 2. Reaction of hydroxyl radical with guanine leads to formation of 8-hydroxyguanine and 2,6-diamino-4-hydroxy-5-formamidopyrimidine.

garding which methods are best to determine DNA oxidation. Recent comparison of LC/MS and GC/MS in the analysis of 8-OHG from calf thymus DNA showed similar results for the two methods and detection limits on the order of 35 fmol from 1 μg DNA using LC/MS and 3 fmol 8-OHdG from 0.1 μg DNA using GC/MS (17). These data also show that the hydrolysis/derivatization steps necessary for GC/MS do not introduce artifactual oxidation of DNA. In addition, further protection against artifactual oxidation has been achieved by excluding oxygen from reaction vials during hydrolysis/derivatization for GC/MS. Our most recent studies show that use of 8-hydroxyquinoline as an antioxidant during phenol extraction also helps limit artifactual oxidation. In direct comparison of levels of 8-OHG from DNA isolated using phenol extraction or prepared using the NaI salting out method, we observed no significant differences in 8-OHG (57). In general, our studies suggest that GC/MS with selective ion monitoring (GC/MS-SIM) and stable-labeled internal standards provide a highly selective and sensitive method in the identification of a wide range of bases simultaneously and unequivocally in a single run (57–58).

The accumulation of nDNA and mtDNA damage from oxidation results from an imbalance between the rate of oxidation and DNA repair mechanisms. To counteract the multiple environmental, physical, and chemical factors causing DNA damage, three DNA repair pathways have evolved. The most prominent repair pathways are base excision repair, nucleotide excision repair, and mismatch repair. Base excision repair is characterized by the excision of nucleic acid base residues in the free form that contain lesions including oxida-

tive damage, alkylating adducts, and deamination products. Nucleotide excision removes damaged nucleotides as part of fragments up to 30 bases in length. Mismatch repair corrects single misrepaired nucleotides and smaller loops.

OXIDATIVE DAMAGE TO DNA IN ALZHEIMER'S DISEASE

The study of oxidative DNA damage and repair in the brain in AD is in its infancy. In 1990, Mullaart *et al.* (48) described a twofold increase in DNA strand breaks in the brain in AD. One of the consequences of DNA strand breaks is the activation of poly (ADP-ribose) polymerase (PARP), a zinc-finger DNA binding protein (33). Overactivation of PARP in response to oxidative damage causes depletion of intracellular NAD^+ that results in depletion of energy stores and cell death (60). Love *et al*. (34) demonstrated increased PARP activity in the frontal and temporal lobes in AD using immunohistochemistry. In another study (55), increased strand breaks determined by terminal deoxynucleotidyl transferase labeling were recorded in non-NFT containing neurons and were associated with prominent nitrotyrosine labeling, which suggests that neurons without NFT but with DNA damage may undergo degeneration by oxidative mechanisms involving peroxynitrite.

A progressive age-related increase in oxidative damage to DNA as shown by increased levels of 8-OHG in nuclear and mitochondrial fractions from three brain regions of cerebral cortex and cerebellum from 10 normal controls ages 42–97 years was reported by Mecocci *et al.* (42). They used HPLC with electrochemical detection in their study, and the rate of increase in aging was much greater in mtDNA. In a similar study of the brain of 13 AD and 15 age-matched controls, the same group showed a threefold increase in 8-OHG in mtDNA in the parietal lobe in AD. The oxidative damage in nDNA was not significantly elevated in AD.

Lyras *et al.* (36) used GC-MS to describe increased levels of 8-OHG, 8-OHA, and 5-OHC in total DNA in the parietal lobe in AD compared to controls. They also described elevated thymine glycol, fapy-adenine, fapy-guanine, and 5-hydroxyuracil (5-OHU), a degradation product of cytosine, in various brain regions in AD. Several oxidized bases were higher in some brain regions in controls compared to AD patients. The mean postmortem interval (PMI) ranged from 20 to 46 h and raised the question of whether prolonged PMIs could cause increased oxidation.

In our DNA oxidation studies, we used GC/MS-SIM with specific stable isotope-labeled analogues that yielded selective and sensitive quantification of base adducts. All specimens used in our studies had PMIs between 2 and 4 h, and all patients and controls had been followed longitudinally in our Alzheimer's Disease Center. In our first study of nDNA in AD patients and controls, we found statistically significant elevations of 8-OHG, 8-OHA, and 5-OHU in temporal, parietal, and frontal lobes in AD, and elevated 5-OHC in temporal and parietal lobes in AD (19). No significant differences were found in fapy-adenine or fapy-guanine. In a subsequent study of nDNA and mtDNA, we found 8-OHG, 5-OHU, 5-OHC, and fapy-adenine significantly elevated in mtDNA in parietal and temporal lobes of AD patients. 5-OHC was also elevated in the AD frontal lobe in mtDNA (58). Nuclear DNA showed significant elevations of 8-OHG, 8-OHA, 5-OHC, 5-OHU, and fapy-adenine in a variable pattern in the neocortex in AD. Overall, mtDNA had approximately 10-fold higher levels of oxidized bases than nDNA. These studies indicate that nDNA and mtDNA undergo considerable oxidative damage in AD and may contribute to the neurodegeneration in AD. All of these studies were on patients with advanced AD and it is not clear whether these changes are secondary to the neurodegenerative alterations of advanced AD.

Recently, we studied nDNA and mtDNA in multiple brain regions in 8 longitudinally followed MCI patients and 6 longitudinally followed control subjects who had short PMI autopsies (57). We found statistically significant elevations of 8-OHG in nDNA in frontal and temporal lobes in MCI and in mtDNA from the temporal lobe of MCI patients compared to age-matched controls. Levels of 8-OHA and fapy-adenine were significantly elevated in nDNA in frontal, temporal, and parietal lobes and fapy-adenine in mtDNA was significantly elevated in these three lobes in MCI. These findings suggest that oxidative damage to nDNA and mtDNA occurs in the earliest detectable phase of AD and is less likely to be secondary to severe neurodegenerative alterations. They also suggest that oxidative damage of DNA may play a meaningful role in the pathogenesis of this disease.

Little is known about the repair of individual products of oxidative damage in the brain. Hermon *et al.* (27) found increased excision repair-cross-complementing gene products 80 and 89, representative of repair genes, in five brain regions in Down syndrome and AD. The AD patients' mean age was 58.7 years. The authors interpreted these findings to be related to ongoing oxidative DNA damage. In nuclei, the repair of 8-OHG is specifically carried out by the base excision repair enzyme, 8-oxoguanine glycosylase (OGG1). OGG1 recognizes the adduct and catalyzes the hydrolysis of the N-glycosylic bond, which links the damaged base to the deoxyribose–phosphate backbone of DNA and results in an apurinic site. The repair of the apurinic site requires apurinic endonuclease, DNA polymerase B, and DNA ligase 3 (5). We studied OGG1 activity and found it significantly decreased in nuclear protein fractions of the hippocampus, superior and middle temporal gyri, and inferior parietal lobule in AD compared to age-matched controls (35). Iida *et al*. (28) used an antibody against the mitochondrial form of OGG1 (hOGG1–2a) and showed decreased expression in NFT-containing neurons in the orbitofrontal cortex and entorhinal cortex in AD compared to control cases. Together those studies suggest an increase in oxidation of guanine and diminished activity of the enzyme that removes oxidized guanine in AD.

Repair of DNA double-strand breaks is by a nonhomologous end joining mechanism that uses the DNA-dependent protein kinase (DNA–PK) complex. Shackelford (53) demonstrated that end joining activity and protein levels of DNA–PK catalytic subunits are significantly lower in AD midfrontal cortex compared to normal controls. This indicates that repair of DNA double-strand breaks may be deficient in AD.

In studies of peripheral DNA oxidation, Mecocci *et al*. (43, 44), using HPLC with electrochemical detection, showed in-

creased 8-OHG levels in lymphocytes in patients with AD compared to controls. In the latter study, they found a decrease in plasma levels of multiple antioxidants that they gave as an explanation for the increased DNA oxidation. Several other studies have detected increased oxidative DNA damage to lymphocytes of AD patients using the comet assay. Morocz *et al*. (47) showed significant elevation of oxidized purines, and Kadioglu *et al*. (31) showed elevated levels of oxidized pyrimidines and purines in DNA of peripheral lymphocytes from AD patients compared to controls. These studies are of interest. However, it is difficult to relate DNA oxidative damage in peripheral lymphocytes to alterations in the central nervous system. Although there have been multiple studies on erythrocytes, lymphocytes, platelets, and skin fibroblasts in AD, there has never been definitive proof that it is a generalized systemic disease.

CONCLUDING REMARKS

Multiple studies have shown an increase in oxidation of nDNA and mtDNA in the brain in AD. In addition, several studies have suggested diminished repair of 8-OHG in nDNA. Importantly, our more recent study has shown increased nDNA and mtDNA oxidation in MCI, the earliest detectable phase of AD. This suggests that it is not a secondary event to degenerative changes in the brain but may contribute to the neurodegeneration in AD. Although it appears that oxidation of DNA plays a role in the pathogenesis of AD, considerably more research must be performed to clarify the mechanisms of DNA oxidation in the disease process.

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ABBREVIATIONS

AD, Alzheimer's disease; Aβ, beta amyloid; DNA-PK, DNA-dependent protein kinase; fapy-adenine, 4,6-diamino-5-formamidopyrimidine; fapy-guanine, 2,6-diamino-4-hydroxy-5-formamidopyrimidine; GC, gas chromatography; hOGG1–2a, mitochondrial 8-oxoguanine glycosylase; HPLC, high pressure liquid chromatography; LC, liquid chromatography; MCI, mild cognitive impairment; MS, mass spectrometry; mtDNA, mitochondrial DNA; nDNA, nuclear DNA; NFT, neurofibrillary tangles; NP, neuritic plaques; OGG1, 8-oxoguanine glycosylase; 8-OHA, 8-hydroxyadenine; 5-OHC, 5-hydroxycytosine; 8-OHG, 8-hydroxyguanine and 8-hydroxyguanosine; 5-OHU, 5-hydroxyuracil; PARP, poly (ADP-ribose) polymerase; ROS, reactive oxygen species.

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