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Expression of 8-oxoguanine DNA glycosylase is reduced and associated with neurofibrillary tangles in Alzheimer's disease brain

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Abstract Recent studies have confirmed the role of reactive oxygen species in the pathogenesis of Alzheimer's disease (AD). 8-Oxo-2'-deoxyguanosine accumulation in AD brain has been discussed, but few studies of DNA repair enzymes in AD brain have been done. Further, a relationship between mitochondrial function and oxidative stress has been noticed. In this study, to evaluate the repair mechanism for oxidative DNA damage in AD brain, we investigated brain tissues from autopsy cases of AD and control cases using an antibody against the mitochondrial form of 8-oxoguanine DNA glycosylase (hOGG1-2a), an enzyme that repairs 8-oxo-2'-deoxyguanosine. hOGG1-2a is expressed mainly in the neuronal cytoplasm in both AD and control cases in regionally different manners. Expression of hOGG1-2a is decreased in the orbitofrontal gyrus and entorhinal cortex in AD compared to that in control cases. Immunoreactivity to hOGG1-2a is associated with neurofibrillary tangles, dystrophic neurites and reactive astrocytes in AD. Our results indicate that the repair enzyme for oxidative damage in mitochondrial DNA may not function appropriately in AD, and thus oxidative DNA damage in mitochondria may be involved in the pathomechanism of AD.

Keywords Alzheimer's disease · Oxidative DNA damage · 8-Oxo-2'-deoxyguanosine · 8-Oxoguanine DNA glycosylase (hOGG1) · Immunohistochemistry

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Introduction

Oxidative damage to DNA may play a role in both normal aging and neurodegenerative diseases [21]. Reactive oxygen species (ROS) are continually formed in vivo by normal cellular metabolism and oxidize proteins, lipids and nucleotides. Recent studies have provided evidence that supports the role of ROS in neuronal degeneration [17, 18, 20, 24] and have demonstrated that several mechanisms related to oxidative stress and free-radical reactions may play a crucial role in the pathogenesis of Alzheimer's disease (AD), specifically, in the formation of senile plaques and neurofibrillary tangles (NFTs) [15, 25, 31, 32, 34, 35]. While an increase of an oxidized nucleotide, 8-oxo-2'-deoxyguanosine (8-oxo-dG), as a marker of DNA damage in AD brain has been reported [7, 13, 18, 21], a decrease in the repair capabilities for 8-oxoguanine in AD brain was also demonstrated [14]. Further, a relationship between mitochondrial function and oxidative stress is implied [9]. Mitochondrial DNA is particularly sensitive to oxidative damage. Since mitochondria are thought to generate most of the free radicals in a cell [2, 21], they could be a major source of ROS that causes oxidative damage to the cell. In addition, the relationship between oxidative damage and β-amyloid protein has been also investigated [3, 4, 10, 28, 42, 43].

There are various protective mechanisms in vivo against oxidative damage by ROS. In *Escherichia coli*, there are three enzymes, MutM, MutY and MutT, that are known to repair errors in DNA caused by 8-oxoguanine [16, 22, 37, 39]. MutM protein excises 8-oxoguanine paired with cytosine in DNA and initiates base excision repair [39], and MutY protein excises adenine paired with 8-oxoguanine in DNA [22]. MutT protein hydrolyzes 8-oxo-dGTP to 8-oxo-dGMP, thereby preventing misincorporation of 8-oxo-dGTP into DNA [1, 16, 33]. We have demonstrated that human cells are also equipped with such protective mechanisms [26, 33]. The human OGG1 gene encodes an 8-oxoguanine DNA glycosylase (a functional homologue of MutM protein), and there are more than seven

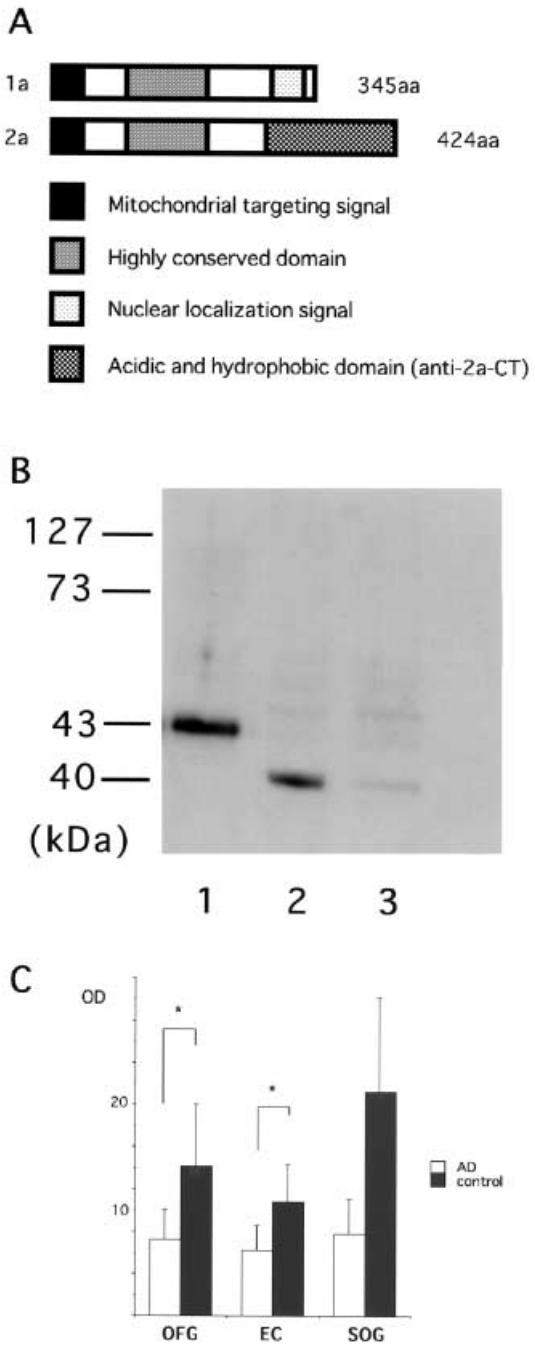

Fig. 1 **A** Structural features of human OGG1 proteins. Both forms of the OGG1 proteins contain a putative mitochondrial targeting signal at the common N-terminal region. There is a nuclear localization signal in the C-terminal end of OGG1-1a alone. Anti-2a-CT recognizes the C-terminal region of OGG1-2a. **B** Immunoblotting of brain homogenates probed with hOGG1 2a-CT antibody. *Lane 1* shows a distinct band at 43 kDa identical to the molecular mass of hOGG1 recombinant protein. *Lanes 2* and *3* show a specific band at 40 kDa identical to molecular mass of the mitochondrial form of hOGG1 protein. *Lane 1* Recombinant hOGG1-2a; *lane 2* control; *lane 3* AD. **C** Optical densities of the OFG, EC and SOG in control and AD cases. Significant decrease of hOGG1-2a optical densities in the OFG and EC of AD is presented as compared to those of control (**P*<0.05). No significant difference of optical density is shown among the OFG, EC and SOG in control and AD cases (*AD* Alzheimer's disease, *OFG* orbitofrontal gyrus, *EC* entorhinal cortex, *SOG* superior occipital gyrus)

spliced forms that are classified into two types based on their last exon (type 1 with exon 7: 1a and 1b; type 2 with exon 8: 2a–2e). Type 1a and 2a mRNAs are frequently found in human tissues, and they share their N termini with the common mitochondrial targeting signal, but each possesses a unique C terminus. The mitochondrial targeting signal at the common N-terminal region of OGG1 proteins is processed at residue 23 (W) after being translocated into mitochondria. hOGG1-1a (36 kDa) is located in the nucleus, while a 40-kDa polypeptide corresponding to a processed form of OGG1-2a is located on the inner membrane of the mitochondria and is specifically detectable using an antibody against its C terminus (2a-CT) (Fig. 1A) [26].

In this study, we immunohistochemically investigated brain tissues from autopsy cases of AD and controls using an antibody against hOGG1-2a to evaluate alteration of the repair mechanism against 8-oxoguanine in AD brains.

Materials and methods

We investigated postmortem human brains (Table 1) from patients with AD (*n*=8, age range 69–95 years, postmortem delay 4–28 h) and patients without neuropsychiatric disorders (*n*=6, age range 72–90 years, postmortem delay 2–23 h). The histological diagnosis of AD followed the guidelines of the Consortium to Establish a Registry for Alzheimer's Disease (CERAD) [23]. Brain tissues were obtained from the Department of Neuropathology, Neurological Institute, Graduate School of Medical Sciences, Kyushu University.

The following antibodies were used in this study. A rabbit polyclonal antibody (anti-2a-CT) recognizing the mitochondrial form of hOGG1 (hOGG1-2a) was raised against fusion proteins containing the amino acid sequence of the C terminus of hOGG1-2a and affinity-purified, as described previously [26]. Mouse monoclonal antibodies to tau (1:1,000 dilution, clone AT8, Innogenetics, Belgium), β-amyloid (1:100 dilution, clone 6F/3D, Dako, Denmark), cytochrome oxidase subunit 1 (1:1,500 dilution, clone 1D6-E1-A8, Molecular Probes, USA) were also used. The β-amyloid antibody recognizes both β-amyloid 40 and 42.

Immunoblotting for hOGG1-2a was performed as follows. Frozen samples of the temporal lobe of an AD brain (case 4) and a control brain (case 1) were separately homogenized in protein lysis buffer in 20 mM TRIS-HCl pH 7.4 containing 10% sucrose and protease inhibitors (protease inhibitor cocktail, Complete Mini, Roche Diagnostics, Switzerland). Each protein sample (10 μg/lane) and recombinant hOGG1 (5 ng) was separated by SDS-polyacrylamide gel electrophoresis on a 12% gel and transferred to a polyvinylidene difluoride membrane (PVDF, Millipore, USA). The membrane was incubated with anti-hOGG1-2a antibody at 4°C overnight, and then washed and incubated with horseradish peroxidase (HRP)-conjugated anti-rabbit IgG antibody (PI1000, 1:15,000 dilution, Vector Laboratories, USA). After washing the membrane, the signal was visualized by enhanced chemiluminescence (Amersham, UK).

Tissue samples were taken from orbitofrontal gyrus (OFG), CA1, CA3, CA4, dentate gyrus (DG), entorhinal cortex (EC), inferior temporal gyrus (ITG), and superior occipital gyrus (SOG) for paraffin sections. The specimens were fixed in 10% formalin and embedded in paraffin. The samples were then sectioned at 5 μm.

Single-label immunohistochemistry for anti-2a-CT, tau, β-amyloid and cytochrome oxidase subunit 1 antibodies was performed on paraffin sections by the indirect immunoperoxidase method. Formic acid treatment was performed with immunohistochemistry for anti-β-amyloid antibody and autoclaving for anti-hOGG1-2a antibody. After incubation with the primary antibodies at 4°C overnight, the sections were subsequently incubated with HRP-conju-

Table 1 Immunoreactivity for hOGG1-2a antibody (*duration* duration of AD in years, *PMD* postmortem delay in hour, *OFG* orbitofrontal gyrus, *DG* dentate gyrus, *EC* entorhinal cortex, *ITG* inferior temporal gyrus, *SOG* superior occipital gyrus, *n* immunoreactivity in NFT, *d* immunoreactivity in dystrophic neurites, *g* fine granular staining in cytoplasm, *r* immunoreactivity in reactive astrocytes, *n.a.* not available)

Case	Age	Sex	Duration	PMD	OFG	CA1	CA3	CA4	DG	EC	ITG	SOG
AD												
1	88	M	1	15	±	$\pm^{nd}$	$+^{n}$	±	–	$+^{nd}$	$+^{nd}$	$++^{g}$
2	89	F	2	28	$+^{nd}$	$+^{nd}$	$+^{nd}$	$\pm^{d}$	–	$+^{nd}$	$+^{d}$	n.a.
3	81	F	3	19	$\pm^{d}$	$\pm^{n}$	±	±	–	$+^{dg}$	$\pm^{nd}$	$+^{dg}$
4	69	F	12	16	±	$\pm^{ndr}$	±	±	–	$\pm^{nd}$	$+^{n}$	$+^{g}$
5	90	F	3	10	±	$\pm^{nd}$	±	$\pm^{r}$	–	$\pm^{nd}$	$\pm^{ndr}$	±
6	95	F	6	4	±	$\pm^{d}$	±	±	±	+	$+^{nd}$	$\pm^{g}$
7	79	F	3	14	±	$+^{nd}$	$\pm^{n}$	$\pm^{d}$	–	$\pm^{nd}$	$\pm^{nd}$	±
8	91	F	3	13	±	$\pm^{n}$	±	$\pm^{n}$	±	$\pm^{nd}$	$\pm^{nd}$	±
Controls												
1	72	F	–	13	+	±	+	+	–	++	+	++
2	80	M	–	7	+	$\pm^{n}$	$+^{n}$	±	–	±	$++^{n}$	+
3	90	F	–	23	++	±	+	±	–	+	+	++
4	76	M	–	16	+	±	±	±	–	+	±	+
5	82	F	–	3	$+^{g}$	±	±	–	–	$+^{n}$	±	$+^{g}$
6	73	M	–	2	++	±	±	–	–	++	+	++

gated secondary antibody (1:200 dilution, Vector Laboratories). The colored reaction product was developed with 3,3'-diaminobenzidine tetrahydrochloride (DAB) solution. The sections were counterstained lightly with hematoxylin. Intensity levels of the immunoreactivity in neuron for hOGG1-2a were evaluated as not detectable (–), faint (±), moderate (+) or strong (++).

Double immunostaining was carried out as follows. After incubation with anti-hOGG1-2a antibodies at 4°C overnight, the sections were incubated with HRP-conjugated streptavidin (1:300 dilution, Amersham), and a brownish reaction product was developed with DAB. The sections were subsequently washed in 0.1 M glycine-HCl buffer, pH 2.2, for 2 h, water for 15 min and TRIS buffer for 30 min. The sections were then incubated with anti-tau or anti-β-amyloid at 4°C overnight. After being washed in TRIS buffer, the sections were incubated with the HRP-conjugated secondary antibody (1:200 dilution, Vector Laboratories). Color development was performed using a mixture of DAB and cobalt chloride in TRIS buffer with H_2O_2, thus producing a dark-blue reaction product.

Double-immunofluorescence staining was also performed and observed with a laser confocal system. Anti-cytochrome oxidase subunit 1 was used as a mitochondrial marker to determine whether the immunostaining of hOGG1-2a corresponded to mitochondria. Combinations of antibodies against hOGG1-2a and tau, or against hOGG1-2a and cytochrome oxidase subunit 1 were used for the primary antibodies. Sections were first incubated with a mixture of the two primary antibodies, which was then followed by Texas red-labeled donkey anti-rabbit IgG and fluorescent isothiocyanate-labeled sheep anti-mouse IgG antibodies (Amersham). These sections were observed under a laser scan microscope (LSM-GB200, Olympus, Japan).

Semiquantitative analysis of immunoreactivity to hOGG1-2a was performed as follows. The intensity of immunoreactivities to hOGG1-2a was evaluated by measuring the optical density. The optical density of an area was determined with NIH image software version 1.6. Sections from the OFG, EC and SOG in all AD and control cases were measured. Four separate fields were selected, and the optical density was obtained for each field. The optical densities were then corrected by subtracting the optical density of the cerebral white matter in the same section for background and averaged. Statistical analysis for the differences of regional change among the OFG, EC and SOG were performed with the Kruskal-Wallis test, using a StatView-J4.5 program (Abacus Concepts, USA). The difference between AD and control cases was statistically evaluated with the Mann Whitney U test; $P<0.05$ was considered significant.

Results

The result of immunoblotting with anti-hOGG1-2a is shown in Fig. 1B. In tissue homogenates from the temporal lobes of AD and control cases, distinct bands at approximately 40 kDa were detected. The 40-kDa polypeptide corresponds to the processed form of hOGG1-2a in mitochondria [26].

The immunohistochemical reactions for hOGG1-2a are presented in Table 1. hOGG1-2a was expressed with a diffuse or fine granular pattern in neuronal cytoplasm in both AD and control cases. In control cases, no significant difference was shown in immunoreactivity among the OFG, EC and SOG (Fig. 1C), although the immunoreactivity in the SOG of controls showed a tendency to be more intense than that of the other regions. The immunoreactivities in the CA1, CA3 and CA4 were less intense than those in the other regions, and in the DG it was almost negative. Seven of eight AD cases showed faint immunoreactivity in the OFG, and only one case showed moderate immunoreactivity. On the other hand, two of six control cases showed strong immunoreactivity, and four of the six cases showed moderate immunoreactivity in the OFG. The immunoreactivities in the EC and SOG of AD also tended to be less intense than those of control cases. Statistical analysis revealed significant decreases in the immunoreactivity of the OFG and EC in AD (Fig. 1C). No significant difference was present in the immunoreactivities among these three regions in AD cases; however, the immunoreactivity in the SOG showed a tendency to be more intense than the others. Fine granular cytoplasmic staining is presented in four of seven AD cases, especially in the occipital lobe (Fig. 2A, B). In AD cases, the immunoreactivities in the CA1, CA3 and CA4 were less than those in the other regions, and in the DG, those were almost negative in AD cases as well as in control cases.

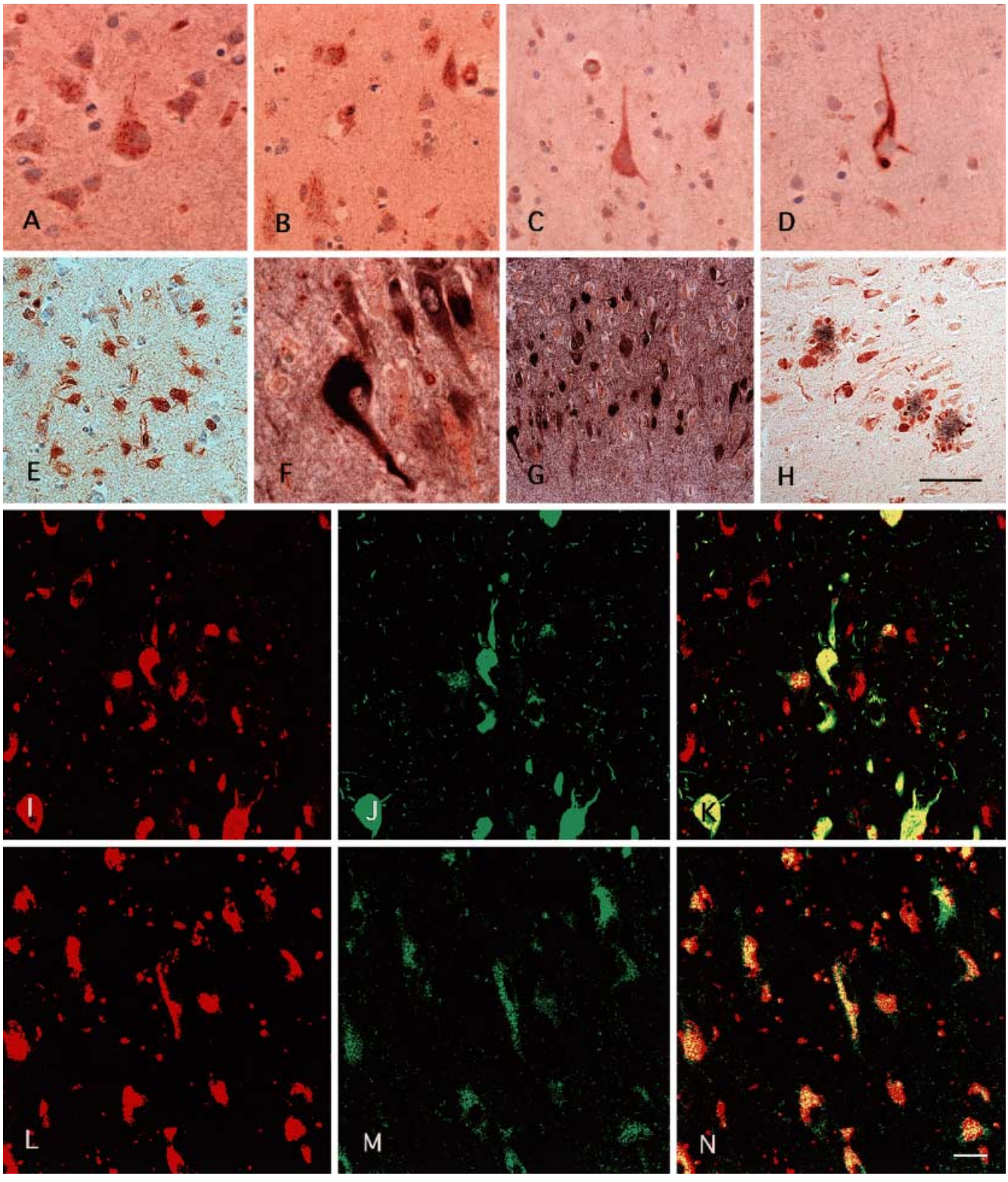

Fig. 2 Immunohistochemistry for hOGG1–2a in AD (**A**–**E**) and double-label immunohistochemistry with hOGG1-2a (*brown*) and tau (*grayish blue*) (**F**, **G**), or with hOGG1 (*brown*) and β-amyloid (*grayish blue*) (**H**) in AD. Double immunofluorescence (*yellow*) labeling with tau (*green*) and hOGG1-2a (*red*) (**I**–**K**), or with cytochrome oxidase subunit 1 (*green*) and hOGG1-2a (*red*) (**L**–**N**). Fine granular staining (**A**, **B**) and diffuse staining (**C**) are present in the cytoplasm of neuron. Reactive astrocytes (**E**) and NFTs (**D**, **F**, **G**) are immunoreactive to hOGG1-2a. Dystrophic neurites around amyloid core are immunoreactive to hOGG1-2a (**H**). NFTs are immunoreactive to both tau and hOGG1-2a (**I**–**K**). Mitochondrial colocalization of hOGG1 and cytochrome oxidase subunit 1 is demonstrated (**L**–**N**). *Bars* **A**, **B**, **D**–**F** 50 μm; **C**, **H** 100 μm; **G** 200 μm; **I**–**N** 30 μm

Immunoreactivity to hOGG1-2a in NFTs and dystrophic neurites was present in all AD cases (Fig. 2D, F–H). Two control cases showed a similar immunoreactivity in NFTs. Reactive astrocytes were also immunoreactive in two cases from AD (Fig. 2E). Double immunostaining with a combination of anti-2a-CT and anti-tau antibodies clearly demonstrated hOGG1-2a immunoreactivity in NFTs (Fig. 2I–K). Double immunostaining with anti-2a-CT and anti-β-amyloid antibodies showed hOGG1-2a-immunoreactive dystrophic neurites around amyloid plaques (Fig. 2H). Double immunostaining with anti-2a-CT and anti-cytochrome oxidase subunit 1 antibodies also demonstrated mitochondrial colocalization of hOGG1 and cytochrome oxidase subunit 1 (Fig. 2L–N).

Discussion

Recent reports have clarified the involvement of oxidative damage to the brain in the development of AD [7, 13, 14, 15, 21, 27]. Superoxide dismutase activity in AD brain has been reported [19], and 8-oxo-dG accumulation in AD brain has been discussed. On the other hand, decreased activity of the repair enzyme for 8-oxo-dG in AD brain has also been reported [14]. We performed immunohistochemistry on AD brains to evaluate the repair mechanism against 8-oxoguanine in mitochondrial DNA. Our results demonstrated regional expression of hOGG1-2a, the mitochondrial form of 8-oxoguanine DNA glycosylase, in brain and decreased hOGG1-2a expression in AD, which may indicate a deficient repair function against oxidative DNA damage in mitochondria. Further, immunoreactivity to hOGG1-2a in NFTs, dystrophic neurites and reactive astrocytes was demonstrated.

The present study disclosed that the mitochondrial type of 8-oxoguanine DNA glycosylase, hOGG1-2a, is expressed in neuronal cytoplasm. The immunoreactivity to hOGG1-2a co-existed with the immunoreactivity to cytochrome oxidase subunit 1; therefore, granular staining of cytoplasm may reflect mitochondrial distribution. Lovell et al. [13] demonstrated an increased level of 8-oxo-dG in DNA extracted from ventricular cerebrospinal fluid and a decreased level of free 8-oxo-dG in ventricular cerebrospinal fluid of AD subjects, and suggested that the brain in AD may be subject to the double insult of increased oxidative stress, and deficiencies in the repair mechanisms responsible for removal of oxidized bases. Moreover, decreased activity of 8-oxoguanine DNA glycosylase in nuclear extracts of examined AD brains was demonstrated [14]. Our result may support this finding. A regional difference in the expression of hOGG1-2a was observed in both control and AD cases. There was no significant difference in its expression among the OFG, EC and SOG in AD; however, the occipital lobe, which is known to be a less affected area in AD, showed a tendency toward more intense expression of hOGG1-2a, and expression varied from strong to faint in AD cases. It may be that these regional changes reflect different phases of the compensatory mechanism for DNA oxidation. Namely, the regions in which immunoreactivity to hOGG1-2a is faint may be in a state in which the repair function for DNA oxidation has exceeded its capacity or is in a less activated state than normal.

The abnormally modified protein known as NFT is one of the most prominent features of AD pathology. Because of its insolubility, NFT has proven difficult to fully characterize and has not completely yielded to biochemical or molecular analysis [8]. Tau has been linked to oxidative stress in numerous studies [12, 30, 38], and immunoreactivity to antioxidant enzymes in NFTs and/or dystrophic neurites of senile plaques has been also reported [6, 29]. Further, oxidative stress products and neuronal nitric oxide synthase in NFTs and senile plaques have been demonstrated [8, 18, 24, 40]. In this study, double immunofluorescence clearly revealed deposition of the repair enzyme for oxidative products in NFTs, and immunoreactivity to hOGG1-2a in NFTs and dystrophic neurites was present in all AD cases. It has been reported that since a small proportion of NFTs are immunoreactive to superoxide dismutase, oxidative stress may follow, rather than precede, development of AD-related neuropathology [29]. On the other hand, Su et al. [36] and Nunomura et al. [27] suggested that nucleic acid oxidation precedes NFT formation. Although the etiology of this pathological process is still unclear, formation of paired helical filaments seems to correlate to DNA oxidation [11, 41]. In addition, hOGG1-2a-immunoreactivity in reactive astrocytes may be associated with immunoreactivity to manganese superoxide dismutase in reactive astrocytes in AD [6]. Taken together, our results and these previous reports may support the hypothesis that oxidative DNA damage affects the formation of NFTs, dystrophic neurites and reactive astrocytes. This is the first report of regional expression of hOGG1-2a, i.e., decreased hOGG1-2a expression in AD brains and immunoreactivity in NFTs, dystrophic neurites and reactive astrocytes. These results indicate that oxidative DNA damage may be involved in the pathogenesis of AD.

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