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Neuronal RNA Oxidation Is a Prominent Feature of Dementia with Lewy Bodies

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ABSTRACT

An *in situ* approach was used to identify the oxidized nucleoside, 8-hydroxyguanosine in brains of dementia with Lewy bodies. Neurons with marked immunoreaction of 8-hydroxyguanosine in the cytoplasm were widely distributed in the hippocampal region and temporal neocortex. Relative intensity measurements of neuronal 8-hydroxyguanosine immunoreactivity showed that there was a significant increase in nucleic acid oxidation in dementia with Lewy bodies compared with controls. Treatment with nuclease (DNase or RNase) before the immunostaining demonstrated that RNA was a major site of nucleic acid oxidation. Together with the previously reported RNA oxidation in vulnerable neurons in Alzheimer and Parkinson diseases, neuronal RNA oxidation in dementia with Lewy bodies might represent one of the fundamental abnormalities in age-associated neurodegenerative diseases.

KEY WORDS: dementia with Lewy bodies; 8-hydroxyguanosine; oxidative stress; RNA ;
synucleinopathy

INTRODUCTION

Several studies have now established the association of neuronal oxidative stress with two major neurodegenerative disorders, i.e., Alzheimer disease (AD) [reviewed in 1, 2] and Parkinson disease (PD) [reviewed in 3, 4], respectively relating oxidative stress with Alzheimer-type as well as Lewy body-type pathology (synucleinopathy). Therefore, it is not surprising that dementia with Lewy bodies (DLB), a clinicopathological entity of accumulation of the protein synuclein, in which Alzheimer-type pathology, a common concurrent feature, is also associated with oxidative stress. Indeed, damage to lipids, proteins, and DNA has been demonstrated in the brains of DLB [5-7].

In this study, we report increased neuronal RNA oxidation by *in situ* labeling of 8-hydroxyguanosine (8OHG), an oxidized nucleoside derived from RNA, in the cerebral cortex of DLB, as we have shown in the cerebral cortex of AD [8] and in the midbrain of PD [9]. Furthermore, by examining levels of RNA oxidation in cases of DLB with and without Alzheimer-type pathology, i.e., “common” and “pure” forms of DLB, we found that both forms of DLB show increased levels of neuronal RNA oxidation indicating an association between synuclein accumulation and oxidative stress.

MATERIALS AND METHODS

Tissue.

Brain tissue was obtained at autopsy from 8 clinically- and pathologically- confirmed cases of DLB (5 males and 3 females; ages 68-85 years, average 76). Two cases (a 68 years old male and a 73 years old male) were subclassified as “pure form” of DLB with no or few senile plaques and neurofibrillary tangles in the neocortical areas, whereas the other 6 cases (3 males and 3 females; ages 72-85 years, average 78) were subclassified as “common form” of DLB with numerous senile plaques and/or neurofibrillary tangles [10]. Data from these cases was compared to data from AD and control groups, i.e., 22 clinically and pathologically confirmed cases of AD (9 males and 13 females; ages 57-93 years, average 78) and a consecutive series of 17 controls without dementia (12 males and 5 females; ages 62-86 years, average 74) [8]. The cases of DLB and AD were not preselected based on any feature analyzed in this study. Postmortem intervals prior to fixation was 2-15 h in DLB, 2-22 h in AD, and 4-23 h in controls. Duration of dementia was known from clinical records in 6 cases of common form of DLB as 3-8 years (average 5.3) as well as in 16 AD cases as 3-16 years (average 8.2). Hippocampal slices (~1cm thick and including the surrounding subiculum, entorhinal cortex and adjacent temporal neocortex) were fixed in methacarn (methanol: chloroform: acetic acid, 6:3:1) for 16 hrs at 4°C, dehydrated through graded ethanol followed by xylene, and embedded in paraffin. Six-micron thick sections were cut, and mounted on Silane® (Sigma, St. Louis, MO)-coated glass slides. From an additional case of pure form of DLB (a 66 years old female), sections of only the pons and cerebellum were examined, as the cerebral cortex was not available.

Immunocytochemistry and Antibodies.

Following deparaffinization with xylene, sections were hydrated through graded ethanol. Endogenous peroxidase activity in the tissue was eliminated by a 30 min incubation with 3% H₂O₂ in methanol and non-specific binding sites were blocked in a 30 min incubation with 10% normal goat serum (NGS) in Tris-buffered saline (150 mM Tris-HCl, 150 mM NaCl, pH 7.6). To detect oxidized nucleosides, we used a mouse monoclonal antibody against 8OHG, 1F7 [11] (1:30; Trevigen, Gaithersburg, MD) after treatment with 10 µg/ml proteinase K (Boehringer Mannheim, Indianapolis, IN) in PBS (pH=7.4) for 40 min at 37°C. Immunostaining was developed by the peroxidase-antiperoxidase procedure [12] by using 0.75 mg/ml 3,3'-diaminobenzidine cosubstrate in 0.015% H₂O₂, 50mM Tris-HCl, pH 7.6 for exactly 10 min. The specificity of 1F7 to 8OHG was confirmed by primary antibody omission or by absorption with purified 8OHG (Cayman Chemical, Ann Arbor, MI) [8]. Although 1F7 recognizes RNA-derived 8OHG as well as DNA-derived 8-hydroxydeoxyguanosine with similar binding affinities [11], we have confirmed that 1F7 immunolabeling in neurons in AD is predominantly in RNA by the pretreatment with DNase or RNase [8] as well as by immunoelectronmicroscopy, which showed most 8OHG is present in the endoplasmic reticulum [13]. For DLB cases, additional sections were pretreated with RNase-free DNase I (10 U/µl for 2 h at 37 °C; Roche, Mannheim, Germany) or DNase-free RNase (0.5 µg/µl for 2 h at 37 °C; Boehringer Mannheim) after the proteinase-K treatment. Lewy bodies were immunostained with rabbit antiserum to α-synuclein (AB5038; 1:1000, Chemicon International, Temecula, CA). Amyloid-β deposits of senile plaque were immunostained with a mouse monoclonal antibody 4G8 (1:1000, Senetek, St. Louis, MO) and neurofibrillary tangles were identified by immunostaining with a mouse monoclonal antibody to phosphorylated τ, AT8 (1:500, Biosource International, Camarillo, CA).

Additionally, sections of six DLB cases were double immunostained with 1F7 and the antiserum to α -synuclein, by using the alkaline phosphatase-antialkaline phosphatase method with fast red substrate-chromogen system (Dako, Carpinteria, CA) and the peroxidase-antiperoxidase method with diaminobenzidine, respectively.

Relative scale of 8OHG

All measurements were performed in stratum pyramidale of prosubiculum adjacent to the CA1 field of hippocampus using a Q500IW-EX Image Processing and Analysis System (Leica) linked to a SONY CCD Camera (XC-75CE) mounted on a Nikon MICROPHOT-FX microscope. The intensity of immunoreaction with 1F7 was evaluated by measuring the average optical density (OD) in an area comprising the cytoplasm and nucleus, as we described previously [8]. Three adjacent fields (each field = $460 \mu\text{m} \times 428 \mu\text{m}$) were selected, and in each field of the video camera, 5 pyramidal neurons sectioned near their equator, based on a section plane that included the nucleolus, were selected and outlined manually so that of the area of the nucleus to cytoplasm was rather constant. The nucleus was included because damage to RNA was nuclear as well as cytoplasmic. The average OD measurement was obtained for each of the 3 fields and averaged. Finally, the OD value was corrected for background by subtracting the OD of the white matter on the same section. All measurements were done under the same optical and light conditions as well as using an electronic shading correction to compensate for any unevenness that might be present in the illumination. Statistical analysis was performed with ANOVA applying Fisher's protected least significant difference as *post hoc* analysis, using StatView 5.0 program (Abacus Concepts, Berkeley, CA).

RESULTS

In cases of DLB, 8OHG immunoreactivity was prominent in the neuronal cytoplasm in the hippocampus, subiculum, and entorhinal cortex as well as temporal neocortex (Fig. 1A, B), as we observed in the brain of AD [8]. Neuronal 8OHG immunoreaction was widely distributed throughout the cortical layers of these regions while in controls, staining was very low (Fig. 1E). Most of the pyramidal neurons in hippocampal CA1 to CA4 fields, subiculum, entorhinal cortex and adjacent temporal neocortex showed similar immunointensity of 8OHG in each case of DLB, although individual variation of the immunointensity among DLB cases was observed. Generally, granular cells in dentate gyrus as well as entorhinal and temporal cortices showed less prominent 8OHG immunoreactivity than pyramidal cells. Among the cases of DLB, not only the common form of DLB but also the pure form of DLB showed positive immunoreaction for 8OHG in several, but not all, of the neuronal populations (Fig. 1B). This was demonstrated for a case of the pure form of DLB where vulnerable neurons in the locus ceruleus showed moderate intracytoplasmic 8OHG immunoreaction while the Purkinje neurons of the cerebellum, that are not effected in DLB, did not (Fig. 1C, D).

Relative scale measurements of the 8OHG immunoreactivity using a computer-assisted image analysis system demonstrated that the increase was significant ($p < 0.01$) in DLB when compared with a control group. Two cases of pure form DLB showed higher 8OHG immunointensity (relative 8OHG = 14.7 and 15.1, arbitrary units) than the maximum 8OHG immunointensity in the control group (relative 8OHG = 9.8). When we compare the 8OHG immunoreactivity in DLB with that of AD [8], there was no significant difference between these two groups (Fig. 2). Levels of the relative 8OHG immunoreactivity were not related to postmortem intervals among DLB cases ($p > 0.1$ by linear regression analysis), as well as among

AD cases or controls [8].

Lewy bodies were not immunoreactive for 8OHG in any of the DLB cases studied. Double immunolabeling for 8OHG and α -synuclein demonstrated that the immunoreactivity of 8OHG in the neuronal cytoplasm was not affected by the presence of Lewy bodies (Fig. 3), which was shown also in the midbrain of PD [9].

To investigate whether the immunoreaction with the 1F7 antibody was derived from oxidized RNA or oxidized DNA or both, we performed nuclease treatment before the immunostaining with 1F7. The immunoreaction in the sections of DLB was diminished greatly by the DNase free-RNase pretreatment but only slightly by the RNase free-DNase pretreatment (Fig. 4), as we demonstrated in the sections of AD [8]. Therefore, not only in AD but also in DLB, RNA is a major site of nucleic acid oxidation.

DISCUSSION

Several types of oxidative damage have been demonstrated in brain tissue from DLB patients, including lipid peroxidation [5], protein carbonyls [6], protein nitration [7], glycooxidation [14], and DNA bases oxidation [6]. However, the findings presented here represent the first evidence of increased oxidative damage to RNA in the cerebral cortex neurons of DLB, a finding previously made for cerebral cortex neurons in AD [8] and in the substantia nigra neurons of PD [9]. Importantly, the increased levels of neuronal RNA oxidation in “pure form” DLB, without Alzheimer-type pathology, indicates that synucleinopathy alone in cerebral cortical neurons is sufficient to be related to oxidative stress. Although hydroxynonenal adducts, nitrated protein, and advanced glycation end-products were demonstrated to accumulate in Lewy bodies themselves [5, 7, 14], 8OHG (oxidized RNA) was not demonstrated in Lewy bodies in this study as well as in the previous study of PD [9], likely because Lewy bodies do not contain abundant RNA species [15].

In vitro studies support important roles of α -synuclein in cellular oxidative balance, i.e., (i) elevated levels of α -synuclein can lead to oxidative stress [16, 17], (ii) aggregation of α -synuclein is the result of oxidative stress [18, 19], and (iii) α -synuclein protects against oxidative stress [20]. These data suggest some remarkable parallels between the ability of α -synuclein and amyloid- β , a key protein of AD pathogenesis, to act as either pro-oxidant or anti-oxidant [21]. In an animal model of PD, chronic treatment with a mitochondrial toxin causes α -synuclein aggregation in the nigral neurons apparently via oxidative stress [22]. Similarly, our *in vivo* study in DLB brains suggests that oxidative damage is not secondary to Lewy body formation or α -synuclein aggregation, because neuronal RNA oxidation occurs widely beyond the distribution of neurons containing Lewy bodies. Moreover, the widespread damage to

neuronal RNA is consistent with the observation that neuronal loss can occur in neuronal populations without the formation of Lewy bodies in synucleinopathy [23]. Interestingly, we observed similar levels of cytoplasmic RNA oxidation among neurons with or without Lewy bodies in the cerebral cortex of DLB, similar to results shown for the substantia nigra of PD [9]. Because nucleolar size, an index of the amount of cellular RNA and RNA histochemistry, is not altered by the presence of Lewy bodies in neurons [24], it can be concluded that the levels of both neuronal RNA content and neuronal RNA oxidation in DLB and PD are not affected by the presence of Lewy bodies. Therefore, in synucleinopathies, neuronal oxidative damage appears to be primary to α -synuclein aggregation and sustained after the α -synuclein aggregation.

CONCLUSION

We observed significantly increased nucleic acid oxidation marked by 8OHG immunoreactivity in both the “common form” and the “pure form” of DLB compared to controls. The 8OHG was mainly restricted to cytoplasmic RNA of vulnerable neurons in DLB. Widespread occurrence of neuronal RNA oxidation beyond the distribution of Lewy bodies may indicate the temporal primacy of oxidative stress to α -synuclein aggregation. Together with the RNA oxidation in vulnerable neurons in AD and PD [8, 9], neuronal RNA oxidation in DLB may represent one of the fundamental abnormalities in age-associated neurodegenerative diseases. Indeed, oxidatively modified RNA bases may lead to errors in protein synthesis compromising the accuracy of transcription and translation [25] that may in turn precipitate protein alterations.

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FIGURE LEGENDS

Fig. 1. Oxidized nucleoside, 8OHG, is abundant in vulnerable neurons in DLB. Neuronal 8OHG immunoreactivity showing cytoplasmic predominance is prominent in the temporal neocortex from a case of common DLB (84 years old) (A) as well as in the hippocampal subiculum from a case of pure DLB (68 years old) (B). In a case of pure DLB (66 years old), moderately positive 8OHG immunoreactivity is observed in neurons (arrowheads) in the locus ceruleus (C), but not in the Purkinje neurons of the cerebellum (D). Figs C and D are taken from the same section. In a control case (80 years old), the neuronal 8OHG immunoreactivity is faint in the hippocampal subiculum (E). Scale bars, 50 μ m.

Fig. 2. Relative scale of 8OHG immunoreactivity in the hippocampal subiculum neurons of 17 controls, 8 cases of DLB, and 22 cases of AD [ref. 8]. Values shown are the means with SE. The differences between DLB and controls as well as between AD and controls are significant ($p < 0.01$ and $p < 0.0001$, respectively), but the difference between DLB and AD is not significant by ANOVA with *post hoc* Fisher's protected least significant difference.

Fig. 3. Double immunolabeling for an oxidized nucleoside, 8OHG (red) and α -synuclein (brown) counterstaining with hematoxylin in the temporal neocortex of a common DLB case (74 years old). The intensity of 8OHG immunoreaction in the neuronal cytoplasm is not affected by the presence of Lewy bodies (arrowheads). Scale bar, 50 μ m.

Fig. 4. RNA is a major site of nucleic acid oxidation in DLB. The immunoreaction with 1F7 antibody in DLB (A) is diminished greatly by the treatment with DNase-free RNase (B) but only

slightly by the treatment with RNase-free DNase (C). *indicates landmark blood vessel in adjacent serial sections. The temporal neocortex from a case of common DLB (82 years old). Scale bar, 50 μ m.







