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Intraneuronal amyloid β accumulation and oxidative damage to nucleic acids in Alzheimer disease

Akihiko Nunomura [a,*], Toshio Tamaoki [a], Koich Tanaka [a], Nobutaka Motohashi [a], Masao Nakamura [b], Takaaki Hayashi [c], Haruyasu Yamaguchi [d], Shun Shimohama [e], Hyoung-gon Lee [f], Xiongwei Zhu [f], Mark A. Smith [f], George Perry [g]

[a] Department of Neuropsychiatry, Interdisciplinary Graduate School of Medicine and Engineering, University of Yamanashi, 1110 Shimokato, Chuo, Yamanashi 409-3898, Japan
[b] Department of Chemistry, Asahikawa Medical College, Asahikawa 078-8510, Japan
[c] Hokkaido Institute of Public Health, Sapporo 060-0819, Japan
[d] Gunma University School of Health Sciences, Maebashi 371-8514, Japan
[e] Department of Neurology, School of Medicine, Sapporo Medical University, Sapporo 060-8543, Japan
[f] Department of Pathology, Case Western Reserve University, Cleveland, OH 44106, USA
[g] College of Sciences, University of Texas at San Antonio, San Antonio, TX 78249-0661, USA

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ABSTRACT

In an analysis of amyloid pathology in Alzheimer disease, we used an *in situ* approach to identify amyloid-β (Aβ) accumulation and oxidative damage to nucleic acids in postmortem brain tissue of the hippocampal formation from subjects with Alzheimer disease. When carboxyl-terminal-specific antibodies directed against Aβ40 and Aβ42 were used for immunocytochemical analyses, Aβ42 was especially apparent within the neuronal cytoplasm, at sites not detected by the antibody specific to Aβ-oligomer. In comparison to the Aβ42-positive neurons, neurons bearing oxidative damage to nucleic acids were more widely distributed in the hippocampus. Comparative density measurements of the immunoreactivity revealed that levels of intraneuronal Aβ42 were *inversely* correlated with levels of intraneuronal 8-hydroxyguanosine, an oxidized nucleoside ($r = -0.61$, $p < 0.02$). Together with recent evidence that the Aβ peptide can act as an antioxidant, these results suggest that intraneuronal accumulation of non-oligomeric Aβ may be a compensatory response in neurons to oxidative stress in Alzheimer disease.

Introduction

Amyloid plaques, filamentous deposits of the amyloid-β (Aβ) peptide in the extracellular space, are defining lesions in Alzheimer disease (AD) brain. The accumulation of Aβ has been demonstrated to occur within neurons prior to extraneuronal deposition of Aβ plaques in patients with Down syndrome, an example of Alzheimer-type neurodegeneration (Gyure et al., 2001; Mori et al., 2002), and in transgenic mouse models of AD (Wirths et al., 2001; Oddo et al., 2003; Lord et al., 2006; Oakley et al., 2006). In AD, intraneuronal Aβ accumulation is evident at early-stages of the disease, including a prodromal stage characterized by mild cognitive impairment (MCI), and subsequently tends to decrease with the emergence of more prominent extraneuronal plaque pathology (Gouras et al., 2000; 2005). Thus, intraneuronal Aβ accumulation is a significant early-stage event in AD.

In contrast, oxidative stress and oxidative cellular damage have been reported to be one of the earliest events in vulnerable neurons of AD (Nunomura et al., 2001; 2006; 2009). Indeed, oxidative damage can be detected prior to the extraneuronal deposition of Aβ plaques in patients with Down syndrome (Nunomura et al., 2000) and in transgenic mice or knock-in mouse models of AD (Pratico et al., 2001; Anantharaman et al., 2006; Resende et al., 2008). In AD and Down syndrome, oxidative damage is more prominent in patients with shorter disease duration and with lesser amount of extraneuronal deposition of Aβ (Cuajungco et al., 2000; Nunomura et al., 2000; 2001; 2004), and is present in brains of subjects with MCI (Ding et al., 2005; Keller et al., 2005; Markesberry et al., 2005; Butterfield et al., 2006; 2007; Wang et al., 2006; Lovell and Makesberry, 2008). Correspondingly, oxidative damage is a significantly early event in AD that is potentially correlated to the intraneuronal Aβ accumulation that characterizes the disease.

Experimentally, Aβ can be used to induce oxidative stress (Behl et al., 1994; Hensley et al., 1994; Schubert et al., 1995; Mark et al., 1997; Tabner et al., 2005), and, on the other hand, oxidative stress can induce Aβ production and accumulation (Yan et al., 1995; Frederikse et al., 1996; Paola et al., 2000; Misonou et al., 2000; Tong et al., 2005; Tamagno et al., 2005; 2008; Shen et al., 2008). However, whether Aβ accumulation is primary to oxidative stress or oxidative stress is primary to Aβ accumulation is yet undetermined in AD. In this study, we investigated the distribution of and relationship between the

* Corresponding author.
E-mail address: anunomura@yamanashi.ac.jp (A. Nunomura).
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levels of intraneuronal Aβ accumulation and oxidative damage to nucleic acids in AD brains. We focused on nucleic acid oxidation because of its pathogenic significance in neurodegeneration (Nunomura et al., 1999; 2001; 2004; 2006; Zhang et al., 1999; Klein et al., 2002; Shan et al., 2003; 2007; Shan and Lin, 2006; Chang et al., 2008) and its utility as a sensitive "steady-state" marker of oxidative stress (Nunomura et al., 2007; 2009) that reveal the chronological relationship between intraneuronal Aβ accumulation and oxidative damage. Altogether, we found more widespread distribution of neuronal oxidative damage compared with intraneuronal Aβ accumulation as well as an *inverse* relationship between them suggesting a possible scenario in which intraneuronal oxidative stress may elicit Aβ in AD as a compensatory measure.

Materials and methods

Tissue

Brain tissue was obtained at autopsy from 16 clinically and pathologically confirmed cases of AD (5 males and 11 females; ages 65–93 years, average 81) according to the National Institute on Aging (NIA) and the Consortium to Establish a Registry for Alzheimer's Disease (CERAD) criteria (Khachaturian, 1985; Mirra et al., 1991). Postmortem intervals prior to fixation were 3–37 h (average 8 h). Hippocampal slices (~1 cm thick and including the surrounding subiculum, and entorhinal cortex) were fixed in methacarn (methanol/chloroform/acetic acid, 6:3:1) for 16 h at 4°C, dehydrated through graded ethanol followed by xylene, and embedded in paraffin. Sections were cut 6 μm thick and mounted on Silane® (Sigma, St. Louis, MO)-coated glass slides.

Immunocytochemistry and antibodies

Following deparaffinization with xylene, the sections were hydrated through graded ethanol. Endogenous peroxidase activity in the tissue was eliminated by a 30-min incubation with 3% H_2O_2 in methanol and non-specific binding sites were blocked in a 30-min incubation with 10% normal goat serum in Tris-buffered saline (150 mM Tris–HCl, 150 mM NaCl, pH 7.6).

To detect Aβ accumulation, we used the following primary antibodies: rabbit polyclonal antibodies, QCB40 (1:100; QCB-Biosource International, Camarillo, CA) and QCB42 (1:250; QCB-Biosource International) raised against the carboxyl terminus of Aβ1-40 (Aβ40) and the carboxyl terminus of Aβ1-42 (Aβ42), respectively; a rabbit polyclonal antibody against Aβ42, AB5078P (1:250; Chemicon, Temecula, CA); and mouse monoclonal antibodies against Aβ42, 8G7 (1:100; Calbiochem, La Jolla, CA), and MBC42 (1:1000; H. Yamaguchi). All of the carboxyl-terminal-specific antibodies against Aβ were well characterized previously and reported to have no or negligible cross-reactivity to full-length amyloid β protein precursor (AβPP) (Gouras et al., 2000; D'Andrea et al., 2001; Kamal et al., 2001; Mori et al., 2002; Takahashi et al., 2004). We also used a rabbit polyclonal antibody specific to the Aβ-oligomer (1:250; gift of Dr. C. Glabe) that was well characterized previously (Kayed et al., 2003). For immunocytochemical detection of Aβ with all the antibodies used in this study, the sections were pretreated with 70% formic acid for 5 min. Of note, it was reported that formic acid pretreatment had little effect on the immunostaining with the conformation-dependent Aβ-oligomer antibody (Kayed et al., 2003, see Supplementary materials).

For the detection of oxidized nucleoside, 8-hydroxyguanosine (8OHG), we used a mouse monoclonal antibody, 1F7 (Yin et al., 1995) (1:30; Trevigen, Gaithersburg, MD), after treatment of sections with 10 μg/ml proteinase K (Boehringer Mannheim, Indianapolis, IN) in phosphate-buffered saline (pH 7.4) for 40 min at 37°C. The specificity of 1F7 for 8OHG was confirmed by primary antibody omission or by pre-absorption with purified 8OHG (Cayman Chemical, Ann Arbor, MI) (Nunomura et al., 1999).

Immunostaining was detected by the peroxidase–antiperoxidase procedure (Sternberger, 1986) using 0.75 mg/ml 3,3′-diaminobenzidine (DAB) co-substrate in 0.015% H_2O_2, 50mM Tris–HCl, pH 7.6 for exactly 10 min. Additionally, sections of several AD cases were double immunostained with the Aβ42 antibody (QCB42) and the 8OHG antibody (1F7), using the alkaline phosphatase–antialkaline phosphatase method with fast red chromogen (Dako, Carpinteria, CA) producing a red reaction product and the peroxidase–antiperoxidase method with nickel-enhanced DAB chromogen (Vector Laboratories, Burlingame, CA) producing a black reaction product, respectively. Sections were not counter-stained to prevent obscuring visualization of the immunolabeling.

Relative scale of intraneuronal Aβ accumulation and 8OHG

The intensities of immunoreactions of Aβ40 with the QCB40 antibody, Aβ42 with the QCB42 antibody, and 8OHG with the 1F7 antibody were evaluated by measuring the optical density. The optical density in an area comprising the cytoplasm and nucleus was determined with a Q500IW-EX Image Processing and Analysis System (Leica) linked to a SONY CCD Camera (XC-75CE) mounted on a Nikon MICROPHOT-FX microscope, as described previously (Nunomura et al., 1999; 2000; 2001; 2004). Neurons from all 16 AD cases were measured in the following manner: Three adjacent fields (each field = 460 × 428 μm) of stratum pyramidale of prosubiculum adjacent to the CA1 field of hippocampus were selected. In each field, 5 pyramidal neurons sectioned near their equator, based on a section plane that included the nucleolus, were selected and outlined manually so that the area of the nucleus to cytoplasm was constant. The nucleus was included because damage to nucleic acids was nuclear as well as cytoplasmic. The average optical density measurement was obtained for each of the 3 fields and averaged. Finally, the optical density value was corrected for background by subtracting the optical density 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 Mann–Whitney -test and linear regression analysis, using StatView 5.0 program (Abacus Concepts, Berkeley, CA).

Results

An *in situ* approach with antibodies specific to the carboxyl terminus of Aβ42 revealed an accumulation of intraneuronal Aβ42 with moderate to strong immunoreactivities in brain tissue of the hippocampus and the subiculum from subjects with AD. This intraneuronal Aβ42 immunoreaction was especially evident within pyramidal neurons and showed a distinct granular pattern in the perikaryal cytoplasm (Fig. 1A). Compared with the intraneuronal Aβ42 immunoreactivity, relatively little Aβ40 immunoreactivity was found within the same neurons (Figs. 1B and C). There was variability in the degree of intraneuronal Aβ 40 and 42 immunoreactivities among anatomical regions (i.e., relatively higher immunoreactivities in CA1 and subiculum compared with CA4) in an individual as well as among the same anatomical region of different subjects.

The C-terminal-specific antibodies against Aβ42 used in this study, namely, QCB42, AB5078P, 8G7, and MBC42, showed similar patterns and levels of Aβ42 immunoreaction in the neuronal cytoplasm. However, the Aβ42-positive neuronal cytoplasm was not immunoreactive with the Aβ-oligomer-specific antibody at the level of light microscopy (Fig. 2).

Compared with the immunoreaction to Aβ42, the immunoreaction to an oxidized nucleoside, 8OHG, were more widespread through CA1