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Intraneuronal Amyloid β Accumulation and Oxidative Damage to Nucleic Acids in Alzheimer Disease

Akihiko Nunomura¹, Toshio Tamaoki¹, Koich Tanaka¹, Nobutaka Motohashi¹, Masao Nakamura², Takaaki Hayashi³, Haruyasu Yamaguchi⁴, Shun Shimohama⁵, Hyoung-gon Lee⁶, Xiongwei Zhu⁶, Mark A. Smith⁶, and George Perry⁷

¹Department of Neuropsychiatry, Interdisciplinary Graduate School of Medicine and Engineering, University of Yamanashi, Chuo, Yamanashi 409-3898, Japan

²Department of Chemistry, Asahikawa Medical College, Asahikawa 078-8510, Japan

³Hokkaido Institute of Public Health, Sapporo 060-0819, Japan

⁴Gunma University School of Health Sciences, Maebashi 371-8514, Japan

⁵Department of Neurology, School of Medicine, Sapporo Medical University, Sapporo 060-8543, Japan

⁶Department of Pathology, Case Western Reserve University, Cleveland, Ohio 44106, USA

⁷College of Sciences, University of Texas at San Antonio, San Antonio, Texas 78249-0661, USA

Abstract

An *in situ* approach was used to identify amyloid- β (A β) 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 A β 40 and A β 42 were used for immunocytochemical analyses, A β 42 was especially apparent within the neuronal cytoplasm, at sites not detected by the antibody specific to A β -oligomer. In comparison to the A β 42-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 A β 42 were *inversely* correlated with levels of intraneuronal 8-hydroxyguanosine, an oxidized nucleoside ($r = -0.61$, $p < 0.02$). Together with recent evidence that the A β peptide can act as an antioxidant, these results suggest that intraneuronal accumulation of non-oligomeric A β may be a compensatory response in neurons to oxidative stress in Alzheimer disease.

Keywords

Alzheimer disease; 8-hydroxyguanosine; intraneuronal amyloid- β ; nucleic acids; oligomer; oxidative stress

Corresponding Author: Akihiko Nunomura, M.D., Ph.D., Department of Neuropsychiatry, Interdisciplinary Graduate School of Medicine and Engineering, University of Yamanashi, 1110 Shimokato, Chuo, Yamanashi 409-3898, Japan, Tel: +81-55-273-9847, Fax: +81-55-273-6765, anunomura@yamanashi.ac.jp.

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Introduction

Amyloid plaques, filamentous deposits of the amyloid- β (A β) peptide in the extracellular space, are defining lesions in Alzheimer disease (AD) brain. The accumulation of A β has been demonstrated to occur within neurons, which precedes extraneuronal deposition of A β 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 A β accumulation is detected 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 A β 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 A β 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 A β deposition (Cuajungco et al., 2000; Nunomura et al., 2000; 2001; 2004). In addition, oxidative damage is detected 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 Markesberry, 2008). Therefore, oxidative damage is also a significant early-stage event in AD, which suggests a possible connection between oxidative stress and intraneuronal A β accumulation in AD.

Experimentally, A β 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 A β 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 A β accumulation is primary to oxidative stress or oxidative stress is primary to A β accumulation is undetermined in AD. In this study, we investigated the distribution and relationship between the levels of intraneuronal A β 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) as well as its utility as a sensitive “steady-state” marker of oxidative stress (Nunomura et al., 2007; 2009) through which we can address the chronological issue of the relationship between intraneuronal A β accumulation and oxidative damage. In the current study, we found more widespread distribution of neuronal oxidative damage compared with intraneuronal A β accumulation and an *inverse* relationship between them, suggesting a possible scenario in which intraneuronal A β accumulation may be a compensatory response to neuronal oxidative stress in AD.

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). 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 hr 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% H₂O₂ 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); 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 Online Supporting 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% H₂O₂, 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 intensity 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 U-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 A β 42 revealed an accumulation of intraneuronal A β 42 with moderate to strong immunoreactivities in brain tissue of the hippocampus and the subiculum from subjects with AD. This intraneuronal A β 42 immunoreaction was especially evident within pyramidal neurons and showed a distinct granular pattern in the perikaryal cytoplasm (Fig. 1A). Compared with the intraneuronal A β 42 immunoreactivity, relatively little A β 40 immunoreactivity was found within the same neurons (Fig. 1B, C). There was variability in the degree of intraneuronal A β 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 A β 42 used in this study, namely, QCB42, AB5078P, 8G7 and MBC42 showed similar patterns and levels of A β 42 immunoreaction in the neuronal cytoplasm. However, the A β 42-positive neuronal cytoplasm was not immunoreactive with the A β -oligomer-specific antibody at the level of light microscopy (Fig. 2).

Compared with the immunoreaction to A β 42, the immunoreaction to an oxidized nucleoside, 8OHG, were more widespread through CA1 to CA4 pyramidal cells and the dentate gyrus granule cells in the hippocampus (Fig. 3A-D), as well as in the neurons of the subiculum. Double immunolabeling with A β 42 and 8OHG showed that some of the neurons were positive with both A β 42 and 8OHG while other neurons were positive only for 8OHG. Importantly, very few neurons were observed to be immunopositive only for A β 42 (Fig. 3E, F). In the CA1/prosubiculum area, approximately 20-40% of neurons were positive for both A β 42 and 8OHG, 10-20% of neurons are positive only for 8OHG, and only a few percentages of neurons are positive only for A β 42.

When we investigated whether there was a relationship between intraneuronal A β and nucleic acid oxidation, we noticed that some AD cases showed an intense intraneuronal A β 42 immunoreaction and a weak neuronal 8OHG immunoreaction, while others showed a weak intraneuronal A β 42 immunoreaction and an intense neuronal 8OHG immunoreaction. Intraneuronal A β 40 immunoreaction was weak in most of the cases and was independent of the neuronal 8OHG immunoreactivity (Fig. 4A-F). Relative immunointensity measurements by the image-analysis system and linear regression analysis reveal that there was a significant *inverse* relationship between intraneuronal A β 42 and 8OHG ($r = -0.61$, $p < 0.02$) (Fig. 4G), while there was no significant relationship between intraneuronal A β 40 and 8OHG ($r = 0.05$, $p > 0.9$) (Fig. 4H). Of note, neither intraneuronal A β 40 immunointensity ($p > 0.2$) nor intraneuronal A β 42 immunointensity ($p > 0.9$) was associated with postmortem interval; neuronal 8OHG immunointensity was not associated with postmortem intervals, either ($p > 0.2$). In addition, neither intraneuronal A β 40 immunointensity ($p > 0.1$) nor intraneuronal

A β 42 immunointensity ($p > 0.6$) was associated with age of the AD patients; neuronal 8OHG immunointensity was not associated with age of the AD patients, either ($p > 0.6$).

Discussion

As previously shown in postmortem brains of AD and Down syndrome as well as brains of transgenic animal models of AD, we observed neuron-associated A β 42 accumulation and nucleic acids oxidation inside cell soma in the vulnerable regions of the brains of AD. It might be speculated that the intraneuronal A β has a causal role in neuronal oxidative damage, because higher concentrations of A β , in the micromolar range, can lead to oxidative stress in various biological systems (Behl et al., 1994; Hensley et al., 1994; Schubert et al., 1995; Mark et al., 1997; Tabner et al., 2005). However, another line of experiments suggests a primary role for oxidative stress in the production and accumulation of A β *in vitro* (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) and *in vivo* (Bayer et al., 2003; Li et al., 2004; Sung et al., 2004; Dumont et al., 2009). Furthermore, recent studies have indicated that the A β peptide can act as an antioxidant, contingent on its concentration and assembly state, and the relative abundance of concomitant redox-active metals (Walter et al., 1997; Andorn and Kalara, 2000; Kontush et al., 2001; Zou et al., 2002; Bishop and Robinson, 2003; Hayashi et al., 2007; Nakamura et al., 2007), suggesting a scenario in which intraneuronal A β accumulation is a compensatory response to neuronal oxidative stress.

In accordance with the above notion, the present study supports the protective role of A β against oxidative stress. Our observation that neuronal oxidative damage is more widespread than intraneuronal A β 42 may indicate a chronological primacy of oxidative stress to intraneuronal A β 42 accumulation. Moreover, the significant *inverse* relationship between intraneuronal A β 42 and neuronal oxidative damage suggests that intraneuronal accumulation of A β 42 represents a compensatory response to neuronal oxidative stress in AD (Smith et al., 2002). Indeed, there is considerable evidence that picomolar or low nanomolar levels of A β or the monomeric form of A β can be neurotrophic or neuroprotective (Yankner et al., 1990; Luo et al., 1996; Kontush et al., 2001; Zou et al., 2002; Plant et al., 2003) and enhances synaptic plasticity and memory (Puzzo et al., 2008). In the original report documenting the presence of intraneuronal A β by Grundke-Iqbal et al. (1989), the A β immunoreaction in neurons was observed in individuals aged 38-81 years with or without AD pathology. In support of this finding, a recent study has reported a constant level of intraneuronal A β accumulation during adulthood and aging in control human subjects, suggesting that intraneuronal A β accumulation is a physiological phenomenon in normal adult brains (Wegiel et al., 2007). Therefore, intraneuronal A β might exert a fundamentally protective function in the normal aging process and the pathological process of age-associated neurodegeneration. Indeed, we have observed much lower levels of neuronal oxidative damage in control elderly compared with AD patients (Nunomura et al., 1999; 2001; 2004), suggesting successful cellular compensation against oxidative stress in normal aging. To address the issue on the role of intraneuronal A β accumulation before the onset of AD, we have started a further investigation of clinically- and pathologically-classified groups of subjects; i.e., normal control, preclinical AD, and mild cognitive impairment.

Previous studies *in vitro* or in the cerebrospinal fluid and plasma showed that both A β 1-40 and A β 1-42 peptides were efficient antioxidants by reducing formation of metal-inducible reactive oxygen species or metal-inducible lipid peroxidation (Kontush et al., 2001; Zou et al., 2002; Hayashi et al., 2007). However, our *in situ* study of AD brains demonstrated an association of intraneuronal A β 42 accumulation with a reduction of neuronal oxidative damage, but not intraneuronal A β 40 accumulation. Because A β 1-42 is reported to have higher affinity to redox-active copper than A β 1-40 (Kontush, 2001), A β 1-42 may be more efficient antioxidant than

A β 1-40 in the AD affected neurons where redox-active copper is likely involved in the pathophysiology (Atwood et al., 2003). Additionally, we should pay attention to the relative paucity of intraneuronal A β 40 accumulation compared with A β 42 accumulation shown in the present study as well as in previous immunohistochemical studies using C-terminal specific antibodies (Gouras et al., 2000; D'Andrea et al., 2001). More sensitive approaches to detect intraneuronal A β 40 may be required for demonstrating an antioxidant activity of intraneuronal A β 40.

As shown by Western blot analyses in previous studies, the A β 42 antibodies used in this study have selective affinities for the conformational state of A β . Indeed, the QCB42 antibody recognizes both monomeric and oligomeric A β 42 species (Mori et al., 2002), whereas the MBC42 antibody recognizes preferentially A β 42 monomers and some dimers but not oligomers (Takahashi et al., 2004). Therefore, the neuronal cytoplasmic A β 42 observed in this study, detected using the QCB42 and the MBC42 antibodies, but not with the A β -oligomer specific antibody, should be mainly non-oligomeric A β . Although it remains to be addressed which assembly state of A β predominates intraneuronally (LaFerla et al., 2007), the cellular toxicity of oligomeric A β or pre-fibrillar A β is generally accepted (Lambert et al., 1998; Walsh et al., 1999; Haas and Selkoe, 2007). Therefore, oxidative insults in AD may be mediated by oligomeric or pre-fibrillar A β . Indeed, a cell culture experiment demonstrated that A β oligomers can induce neuronal oxidative stress (De Felice et al., 2007). Using an *in situ* approach to the AD brain samples, analysis by immunoelectron microscopy detected immunoreactions of oligomeric A β within dystrophic neuronal processes (Takahashi et al., 2004; Kokubo et al., 2005). At the level of light microscopy, the study by Lacor et al. (2004) showed immunoreactions of oligomer A β , but the oligomers were deposited extracellularly around neurons. The study by Kokubo et al. (2005), however, reported that the immunoreactions of oligomeric A β are not strong enough to be easily recognized at the light microscopic level, which is consistent with our observations. A limitation of our *in situ* approach is that the immunoreactions are not sensitive enough to demonstrate the existence of oligomeric A β . Therefore, our results do not preclude the possibility that oligomeric A β may mediate oxidative damage as a part of assembly state-dependent toxicity of A β .

We previously reported that the area of *extra*-neuronal deposition of A β is inversely associated with levels of neuronal oxidative damage in subjects with AD (Cuajungco et al., 2000; Nunomura et al., 2001; 2004) and Down syndrome (Nunomura et al., 2000). In the current study, we showed for the first time that intraneuronal accumulation of A β , probably in a non-oligomeric form of the peptide, is inversely associated with neuronal oxidative damage. These observations on *extra*- and *intra*-neuronal A β in postmortem brains are exactly in accordance with recent evidence *in vitro* that both monomeric and fibrillar forms of A β act as sacrificial antioxidants by quenching hydroxyl radicals (Nadal et al., 2008). Overall, we can speculate that A β may exert a protective cellular function against oxidative damage and the degree of protection may depend on its assembly state (Kontush, 2001; Atwood et al., 2003; Baruch-Suchodolsky and Fischer, 2008). Clearly, further investigations are required to elucidate the assembly state-dependent properties of A β that potentially mediates cellular toxicity or protective function in relation to redox pathophysiology in AD. These aspects may provide a rationale for pursuing assembly state-specific approaches to A β pathology in establishing efficient therapeutic strategies for AD.

Conclusion

An inverse relationship between intraneuronal immunoreactivities of A β 42 and nucleic acid oxidation has been shown in the hippocampus of AD brains. Intraneuronal accumulation of A β 42 may be involved in a protective cellular mechanism to cope with oxidative insults in AD.

Further investigations are required to understand the assembly state-dependent properties of A β and their roles in redox pathophysiology in AD.

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Fig. 1.

A β 42 and A β 40 immunoreactions in the hippocampus of a 90-year-old patient of AD. Immunoreaction with C-terminal specific antibodies against A β 1-42 (the QCB42 antibody) (**A**, **B**) and A β 1-40 (the QCB40 antibody) (**C**). **A**. In addition to extracellular A β plaques, pyramidal neurons are immunolabeled with A β 42 from CA1 to CA4 in the hippocampus. Granule cells of the dentate gyrus are less immunoreactive. *str. gran.*, stratum granulosum. Scale bar, 500 μ m. **A-inset**. Higher magnification reveals a granular pattern of A β 42 immunoreaction in the perikaryal cytoplasm of the neuron in the hippocampal CA1 region. Scale bar, 10 μ m. **B**, **C**. The hippocampal CA4 region. In adjacent serial sections, perikaryal cytoplasm and dendritic processes of neurons (1-5) are immunopositive for A β 42 (**B**) but not for A β 40 (**C**), while extraneuronal depositions (*arrows*) are positive for both A β 42 and A β 40 (**B**, **C**). *indicates landmark blood vessel. Scale bar, 50 μ m

Fig. 2.

Assembly state specificity of the Aβ antibodies may be a significant determinant of the Aβ immunoreactions. Adjacent serial sections of the hippocampal CA1 area of a 77-year-old AD patient were immunostained with the MBC42 antibody, known to recognize Aβ42 monomers and dimers, but not oligomers (**A**) and with the Aβ oligomer-specific antibody (**B**). Besides extracellular Aβ plaques, intraneuronal Aβ (*arrows*) are immunolabeled with the MBC42 antibody, while there is no specific intraneuronal immunoreaction with the Aβ oligomer-specific antibody (**B**). *indicates landmark blood vessel. Scale bar, 100 μm

Fig. 3.

Accumulation of Aβ42 (the QCB 42 antibody) and nucleic acid oxidation in the hippocampus of a 83-year-old patient of AD. Immunolabeling with Aβ42 (**A, C**) and the oxidized nucleoside, 8OHG (**B, D**) and double immunolabeling with Aβ42 and 8OHG (**E, F**) in adjacent serial sections. **A-D**. Compared to the Aβ42 immunoreactions (**A**), the 8OHG immunoreactions are widespread throughout CA1 to CA4 pyramidal cells as well as granule cells in the dentate gyrus (**B**). **C** and **D** show a high power view of the rectangle area (the CA1 field) in **A** and **B**, respectively. **E, F**. Double immunolabeling with Aβ42 (black) and 8OHG (red). **F** and **F-inset**, high power views of the large and small rectangle areas in **E**, respectively. A large number of the hippocampal CA1 pyramidal neurons are positive for both Aβ42 and 8OHG (dark red neurons, arrow heads), while several neurons are only positive for 8OHG (bright red neurons, arrows). However, very few neurons are only immunopositive for Aβ42 (a black neuron, double arrows in **F-inset**). The black labeling outside neurons is on the diffuse plaques (double asterisks). *indicates landmark blood vessel. Scale bars; **A, B**, 500 μm; **C-F**, 50 μm

Fig. 4.

An inverse relationship between intraneuronal Aβ42 and nucleic acid oxidation. **A-F.** Adjacent serial sections of the hippocampal subiculum immunostained with antibodies against Aβ42 (**A, D**; the QCB 42 antibody), Aβ40 (**B, E**; the QCB 40 antibody), and 8OHG (**C, F**) from two AD cases, a 77-year-old (**A-C**), and an 82-year-old (**D-F**). One case shows an intense intraneuronal Aβ42 immunoreaction (**A**) and a weak neuronal 8OHG immunoreaction (**C**), while the other case shows a weak intraneuronal Aβ42 immunoreaction (**D**) and an intense neuronal 8OHG immunoreaction (**F**). Intraneuronal Aβ40 immunoreaction is weak in both cases (**B, E**). *indicates landmark blood vessels. Scale bar, 50 μm. **G, H** Relative immunointensity measurements (arbitrary units) and linear regression analysis reveal that there is a significant *inverse* relationship between intraneuronal Aβ42 and 8OHG ($r = -0.61$, $p < 0.02$) (**G**), while there is no significant relationship between intraneuronal Aβ40 and 8OHG ($r = 0.05$, $p > 0.9$) (**H**).