Hirosaki Med. J. 61 (Suppl.) : S105—S110, 2010

# OLIGOMERIC Aβ IS THE SOLE CULPRIT MOLECULE TO CAUSE ALZHEIMER'S DISEASE?

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**Abstract** Alzheimer's disease (AD) is the major and common disease usually for aged people to show progressive neurodegenerative disorder with the dementia. Amyloid-beta (also β-protein and referred here to as Aβ) is a well-established seminal peptide in AD that is produced from the amyloid precursor protein (APP) by consecutive digestions with β-secretase of BACE and gamma-secretase of the presenilin complex. Abnormal cerebral accumulation of Aβ such as insoluble fibrils in senile plaques and cerebral amyloid angiopathy (CAA) are observed as a neuropathological hallmark of AD. In contrast to such insoluble fibrillary Aβ, a soluble oligomeric complex is discussed as ADDLs, Aβ oligomer, low-n oligomer Aβ, Aβ*56 or so. Despite their different names, it is proposed as the current hypothesis that oligomeric Aβ is the direct molecule to cause synaptic toxicity and cognitive dysfunction in the early stages of AD. We identified a novel APP mutation (E693delta; referred to as the Osaka mutation) in a pedigree with probable AD resulting in a variant Aβ lacking glutamate at position 22. Based on theoretical prediction and *in vitro* studies on synthetic mutant Aβ peptides, the mutated Aβ peptide showed a unique aggregation property of enhanced oligomerization but no fibrillization. This was further confirmed by PiB-PET analysis on the proband patient. Collectively together, we conclude that the Osaka mutation is the first human evidence for the hypothesis that oligomeric Aβ is involved in AD.

Hirosaki Med. J. **61**, **Supplement** : S105—S110, 2010

**Key words:** Alzheimer's disease; amyloid genetics; oligomeric Aβ

Amyloid beta (Aβ) oligomers are suggested to cause synaptic dysfunction in the early stages of Alzheimer's disease (AD)[1-6]. However, their precise contribution to the AD pathology is unknown: It is not evident whether oligomer formation of Aβ is sufficient for the progression of the disease in the absence of fibril formation. We had a chance to study a patient with a Japanese familial AD who was supposed to have Aβ oligomers without fibrils.

The proband was a 62-year-old woman with a history of suspected familial AD. She noticed memory disturbance at the age of 56. She had no history or symptoms of other neurological disorders. Her Hachinski's ischemic and Mini Mental State Examination (MMSE) scores were normal. MRI and PET showed no cortical atrophy or abnormal metabolism, while SPECT demonstrated bilateral mild hypoperfusion in the temporal lobes. Electroencephalogram showed bilateral intermittent generalized slow theta activity. Thus, she was diagnosed as having mild cognitive impairment at that time. At the age of 59, she showed ideomotor apraxia, and her MMSE score was 22/30 points. According to the Diagnostic and Statistical Manual of

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Mental Disorders (DSM-IIIR) and the criteria of the National Institute of Neurological and Communicative Disorders and Stroke, AD and Related Disorders Association (NINCDS-ADRDA), she was diagnosed as having AD. At the age of 62, her MMSE score dropped to 5 and she exhibited cerebellar ataxia. The axial T1 weighted MRI images showed only mild parietal lobe atrophy. Genetic analysis was examined after an appropriate consultation in which they gave their informed consent to participate in this study. This study was approved by the institutional ethics committee of Osaka City University Graduate School of Medicine. Exons 16 and 17 of APP and all exons of presenilins 1 and 2 were amplified from the patient's genomic DNA by PCR. The DNA sequence of each product was analyzed using a BigDye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems, Tokyo, Japan) and an ABI PRISM 310 genetic analyzer (Applied Biosystems). Since this patient was found to have a mutation in APP exon 17 but not in the presenilin 1 or 2 exons, only APP exon 17 was examined for other family members. ApoE genotyping was performed by detection of the restriction site polymorphism, as described previously[7].

From her and her family members, we identified a novel mutation (hereafter referred to as the Osaka mutation) in APP exon 17 but not in presenilin 1 or 2 exons. This mutation is the deletion of codon 693 encoding glutamate (E693delta) at position 22 in the Aβ sequence. The patient had a homozygous deletion while her unaffected older and younger sisters showed only heterozygous deletions. ApoE genotype appeared not to be associated with this familial case. This Osaka mutation is the first deletion-type mutation in APP. The same homozygous deletion was recently found in another pedigree with AD and the heterozygous deletion in two other pedigrees, one of which was with mild cognitive impairment and the other was normal. These findings strongly suggest that the Osaka mutation is a cause of AD. In addition, this mutation might represent the first recessive one linked to familial AD, though any conclusion cannot be drawn from the little information. To identify Aβ species produced from the mutant APP, we examined the molecular mass of Aβ secreted from HEK293 cells transfected with the APP construct. The resultant Aβ was found to start and end at normal positions but lack a glutamate at position 22. The secretion of the mutant Aβ1-42 and Aβ1-40 were both reduced to about 60% of wild-type Aβ but the ratio A β1-42/ Aβ1-40 was unaffected. This lowered Aβ secretion may explain why the present mutation appears to recessive. This issue remains to be further investigated.

To examined their aggregation property, the mutant Aβ1-40deltaE and Aβ1-42deltaE peptides, which lack a glutamate at position 22, were synthesized (American Peptide Company, Sunnyvale, CA). Molecular weight and amino acid composition of the peptides were confirmed by electro spray mass spectral analysis and amino acid analysis, respectively. The purity of the Aβ1-40deltaE and Aβ1-42deltaE peptides, which was determined by reverse-phase HPLC, was 95.0% and 91.0%, respectively. Control wild-type Aβ1-40 and Aβ1-42 peptides were obtained from American Peptide Company and Peptide Institute (Osaka, Japan). In the thioflavin T fluorescence assay, wild-type peptides showed a quick increase of fibril aggregation, whereas the mutant peptides exhibited little or no increase. In Western blotting, synthetic Aβ peptides were initially dissolved to 0.1 mM in the alpha-helix promoting solvent hexafluoroisopropanol (HFIP) (Sigma) and the solvent was evaporated under vacuum using a Savant Speed Vac system (GMI, Ramsey, MN). The dried peptides were resuspended to 1 mM in 0.1% $NH_4OH$ and dispensed, in quadruplicate, into tubes containing PBS to make a peptide concentration of 100

microM. The peptide solutions were incubated at 37° C for 7 days; aliquots were taken every 24 hr to monitor peptide aggregation by ThT fluorescence assay[8]. For Western blotting, the aliquots were diluted 10-fold in SDS sample buffer and boiled for 5 min. After a further 200-fold dilution in SDS sample buffer, 4 micro-litre (equivalent to 0.2 pmol Aβ peptide) of sample was separated by SDS-PAGE on a 12% NuPage Bis-Tris gel (Invitrogen, Tokyo, Japan) and transferred to Immobilon-P polyvinylidene difluoride (PVDF) membrane (Millipore, Tokyo, Japan). The membranes were boiled in PBS for 10 min to enhance signals and blocked overnight with 3% BSA/1% skin milk/0.1% Tween 20/150 mM NaCl/50 mM Tris-HCl, pH 7.6. Aβ peptides were probed with 6E10 or beta001 followed by horseradish peroxidase (HRP) -labeled anti- mouse or anti-rabbit antibody (Bio-Rad Laboratories, Tokyo, Japan), respectively. Wild-type peptides showed a rapidly decrease of monomers, reflecting their aggregation into fibrils, but those of the mutant peptides only gradually decreased. However, the mutant peptides showed massive formation of SDS-stable oligomers (dimers, trimers and tetramers) immediately after solubilization.

The peptides in aliquots were adsorbed onto 200-mesh Formvar-coated copper grids and negative-stained with 2% uranyl acetate. The specimens were viewed using a JEM-1200EXII electron microscope (JEOL, Tokyo, Japan), showing that wild-type Aβ1-42 peptide formed abundant fibrils during 7-day incubation, whereas virtually no fibrillization was observed in the mutant peptide. Thus, the mutant peptides were shown to rapidly form stable oligomers but not to transform into fibrils.

The unique aggregation property of the mutant Aβ was suggestive of no amyloid formation in the patient's brain. To assess this possibility, we performed PET amyloid imaging of the patient's brain with [$^{11}$C]- Pittsburgh compound-B (PiB) using a PET scanner Eminence-B (Shimadzu Corp., Kyoto, Japan) which was composed of 352 detector blocks, each with a 6 x 8 array of 3.5 x 6.25 x 30 mm$^3$ bismuth germinate oxyorthosilicate crystals, arranged as 32 crystal rings with 208 mm axial field of view. Transmission scans were performed before PiB administration for 5 min in singles mode with $^{137}$Cs point source to obtain attenuation correction data. Emission data were acquired over 60 min (29 frames: 6×30 s, 12×60 s, 5×180 s, 6×300 s). Images were reconstructed with segmented attenuation correction, using Fourier rebinning followed by two-dimensional filtered back-projection applying Ramp filter cutoff at Nyquist frequency. Three-dimensional Gaussian filter with a kernel full-width of a half maximum of 5 mm was applied to the images as a post filter. All subjects had an intravenous bolus injection of 150-300 MBq PiB with a high specific activity (average 20-30 GBq/micro mol). PiB retention data were given as standard uptake values, as described previously[9]. Slight but significant PiB retention signals were observed in temporal, parietal and occipital lobes and cerebellum but not in frontal lobe, which was apparently different

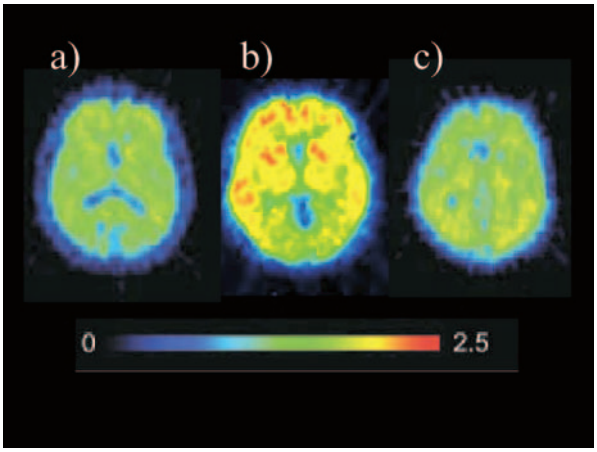


**Figure 1** PiB-PET analysis

Amyloid imaging of the patient's brain with [$^{11}$C]PiB. PiB standardized uptake value images summed over 40 to 60 minutes are shown. (a) aged control (81-yr, female); (b) sporadic AD (71-yr, female); (c), the present case (62-year-old, female).

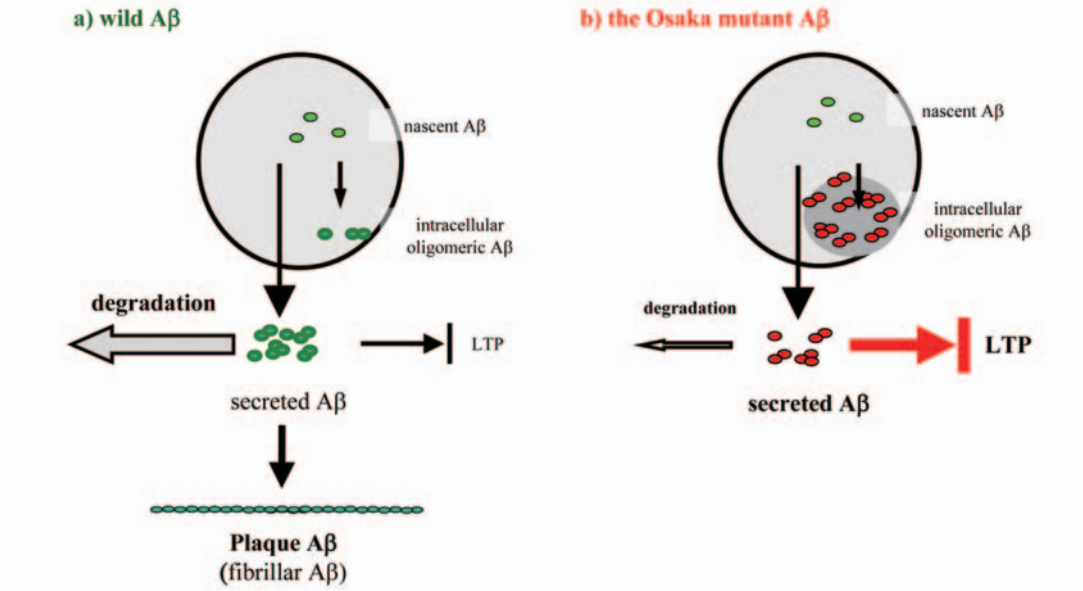


**Figure 2** Scheme of Aβ pathway

Aβ was produced from APP by the consecutive digestions with β-secretase and γ-secretase. (a) Wild Aβ was produced from APP and its majority was secreted out in media. Some of secreted Aβ was in an oligomeric form that causes the synaptic LTP function. (b) The mutant Aβ was enhanced to form an oligomeric state due to the lack of the 22$^{nd}$ glutamate residue resulted in less secretion and in intracellular accumulation. The mutant Aβ was less vulnerable to proteases such as IDE or neprilysin and showed stronger inhibiting activity on LTP than wild Aβ.

from most cases of AD. Thus, the absence of fibril formation of the mutant Aβ was observed both *in vitro* and *in vivo*. It has been proposed that the formation of a beta-turn at positions 22 and 23 in Aβ molecules plays a crucial role in peptide aggregation[10]. The Osaka mutation at position 22 may cause disruption of the secondary structure of the peptide necessary for its formation into fibrils. The lack of a polar amino acid (glutamate) should lead to increased hydrophobicity of the peptide, which may result in accelerated assembly of the peptides into oligomers.

The recent findings of Aβ oligomer-induced synaptic dysfunction[3,4] led us to examine effects of the mutant Aβ on synaptic plasticity in comparison with wild-type Aβ. Synthetic Aβ1-42 peptides were injected into rat cerebral ventricle and hippocampal LTP was examined *in vivo*. As shown previously[3], wild-type peptide caused a significant inhibition of LTP ($p$ = 0.0497 vs. PBS). Notably, the mutant peptide showed a much stronger inhibition than wild-type peptide ($p < 0.0001$ vs. PBS; $p < 0.0001$ vs. wild-type). The observed result appears to reflect the ability of the mutant peptide to form oligomers.

Beside excellular Abeta, several reports have suggested that synaptic dysfunction and neurodegeneration are associated with intraneuronal Aβ[11-17]. We also examined their occurrence in cells transfected with wild and mutated APP cDNA.

Unlike other APP mutations[18-22], the Osaka mutation neither increased total Aβ or Aβ1-42 production nor promoted Aβ aggregation into fibrils but markedly enhanced Aβ oligomerization. Our results suggest that this novel mutation causes AD by enhancing Aβ oligomerization. Furthermore, it is suggested that Aβ fibrillization is not a definite requirement to induce AD, rather its oligomerization may be a crucial event in the pathogenesis of the disease. Alternatively, the present case may represent not a typical but variant type of AD in which the enhanced oligomerization of Aβ enables

to induce the disease without Aβ fibrillization. Finally, as a model to represent Aβ oligomer-related neuropathology, our $APP_{E693delta}$-Tg mice have an advantage in that they control for the influence of Aβ fibrils. Therefore, they will be useful not only for investigating the pathogenic roles of Aβ oligomers but for evaluating therapeutic strategies for AD targeting Aβ oligomers. This will be hoped to break through a current problem on Aβ oligomers. Probably our main goal is to vividly show and specify "the Aβ oligomer" as the culprit of the disease.

## Acknowledgments

This study was supported by Grants-in-aid for Scientific Research from MEXT of Japan to T.T. and H.M., a Health and Labour Sciences Research Grant on Dementia and in part by the Alzheimer's Association (IIRG-09-132098) to H.M.

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