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Review

Visualization of Amyloid Oligomers in the Brain of Patients with Alzheimer's Disease

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In the pathogenesis of Alzheimer's disease (AD), highly neurotoxic amyloid-β (Aβ) oligomers appear early, they are thus considered to be deeply involved in the onset of Alzheimer's disease. However, Aβ oligomer visualization is challenging in human tissues due to their multiple forms (e.g., low- and high-molecular-weight oligomers, including protofibrils) as well as their tendency to rapidly change forms and aggregate. In this review, we present two visualization approaches for Aβ oligomers in tissues: an immunohistochemical (using the monoclonal antibody TxCo1 against toxic Aβ oligomer conformers) and imaging mass spectrometry using the small chemical Shiga-Y51 that specifically binds Aβ oligomers. TxCo1 immunohistochemistry revealed Aβ oligomer distributions in postmortem human brains with AD. Using Shiga-Y51, imaging mass spectrometry revealed Aβ oligomer distributions in the brain of a transgenic mouse model for AD. These two methods would potentially contribute to elucidating the pathological mechanisms underlying AD.

Key words: Alzheimer's disease, amyloid oligomers, senile plaque, immunohistochemistry, imaging mass spectrometry

I. Introduction

Alzheimer's disease (AD) is the most common dementia-related disease accounting for approximately two-thirds of the dementia cases [17, 25]. In order to treat dementia, elucidating the underlying mechanisms of AD pathology is thus essential. The first AD case was reported by Alois Alzheimer. He described the symptoms and neuropathological findings related to the case of a 51-year-old woman and providied careful illustrations of neuritic plaques (senile plaques) and neurofibrillary tangles visualized by silver impregnation staining [2]. Figure 1 shows senile plaques (Fig. 1A, 1B and 1C) and neurofibrillary tangles (Fig. 1D, 1E and 1F) using three different staining methods. The pathological structures cannot be properly visualized by classical staining methods, such as hematoxylin and eosin (HE) (Fig. 1A and D), but by silver impregnation staining (Fig. 1B and E) and immunohistochemistry (Fig. 1C and F). Pathological structure visualization allows for the distinction of healthy aging and dementia as clues to understanding disease pathogenesis. Since the discovery of AD, senile plaques and neurofibrillary tangles in the brain are pathological hallmarks for AD diagnosis and therapeutic targets. In 1984, Glenner and Wong reported the first successful sequencing of amyloid β-peptide (Aβ) from the meningeal blood vessels of patients with AD [7]. A year later, Aβ was detected in the senile plaques in the brain tissue of a patient with AD [20]. In the mid-1980s, paired helical filaments (PHF) were described to constitute neurofibrillary tangles [12]. In 1993, Iqbal *et al.* reported hyperphosphorylated tau proteins as the main PHF components [15]. These findings, along with

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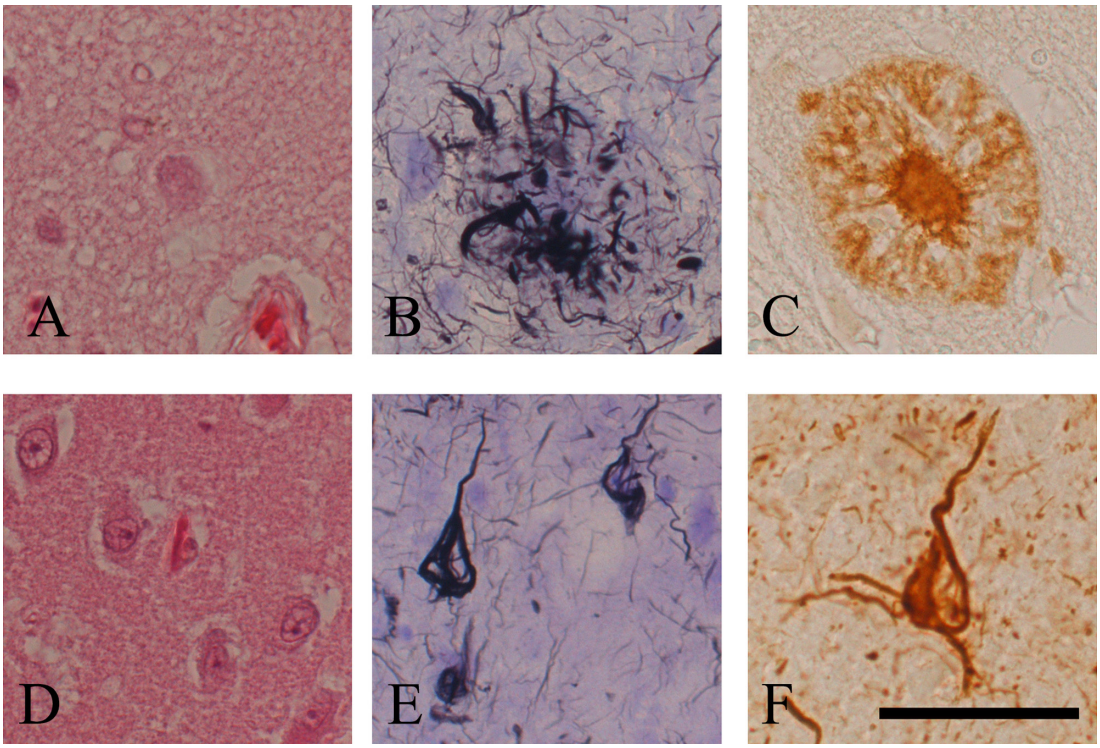

Fig. 1. Histochemistry of senile plaques (**A–C**) and neurofibrillary tangles (**D–F**) in the brain of a patient with Alzheimer's disease using HE (**A** and **D**) and Gallyus (**B** and **E**) stainings as well as immunohistochemistry (**C** and **F**) using the antibody 6E10 against Aβ (**C**) or AT-8 against the phosphorylated tau protein (**F**). Bar = 50 μm.

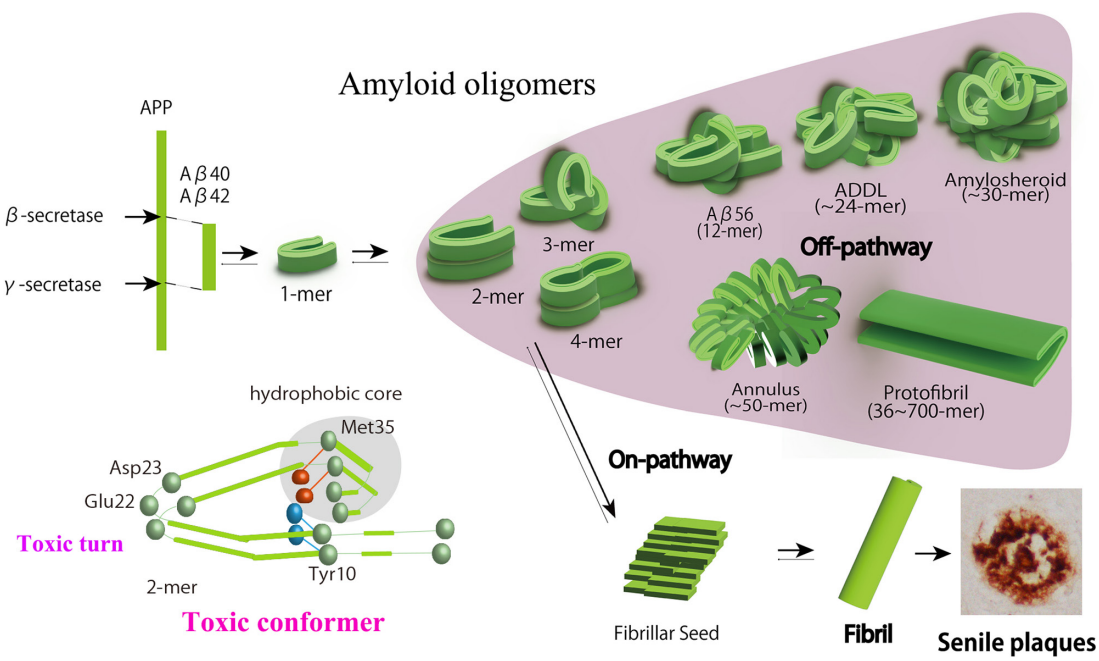

Fig. 2. Schematic drawings of Aβ oligomers. Multiple forms of Aβ oligomers exist in the on- and off-pathway. Toxic Aβ oligomers might display the same basic structure named toxic conformers.

molecular genetic results, led to the amyloid hypothesis [10], serving as theoretical background for developing diagnostic and therapeutic methods [11]. AD research history reveals that the histological detection of pathological structures marks the beginning of the modern research era related to this disease.

II. Aβ Oligomers are the Main Therapeutic Targets for AD

The main component of senile plaques and neurofibrillary tangles are Aβ and hyper-phosphorylated tau protein [17]. The first genetic mutations causing familiar AD were discovered in the amyloid precursor protein (APP) gene [8]. Therefore, the amyloid hypothesis has been formulated, proposing that Aβ deposition is the primary event in AD pathology [10, 11]. According to the amyloid hypothesis, reduction of Aβ production, increase of Aβ clearance and inhibition of Aβ aggregations represent AD therapeutic targets. So far, more than 100 AD drugs have been tested and failed in clinical trials for AD therapy [3]. This raises the question of whether the amyloid hypothesis is correct. Neuropathologists have also reported that the number of senile plaques is not correlated with cognitive decline of AD patients [4].

In the late 1990s, several studies have indicated that the brains of patients with AD contain soluble Aβ assemblies, including Aβ oligomers, and the degree of cognitive impairment correlates with the amount of soluble Aβ oligomers but not the total Aβ burden [16, 18, 22]. Aβ oligomers, rather than monomers or insoluble Aβ fibrils, might be responsible for the neuronal and synaptic dysfunction in AD [5, 31]. This concept is supported by a study describing that Alzheimer-type dementia occurred in a patient with a novel mutation in the APP gene resulting in Aβ oligomers but not senile plaques [28]. The aforementioned findings served as a basis for the amyloid oligomer hypothesis [6] and the idea of focusing on Aβ oligomers as the main therapeutic targets for AD.

Recently, monoclonal antibodies, aducanumab and lecanemab, have been described as efficient agents in treating AD [27, 30]. Interestingly, both monoclonal antibodies could react not only with Aβ fibrils but also with protofibrils, high-molecular-weight Aβ oligomers. The successful antibody application in AD treatment demonstrates the importance of amyloid oligomers. However, to date, no one has succeeded in visualizing Aβ oligomers in the brain of patients with AD. Therefore, we attempted to visualize Aβ oligomers.

III. Immunohistochemical Approach Using Monoclonal Antibodies Against Toxic Conformers of Aβ Oligomer

Aβ oligomers consist of multiple forms such as low-molecular-weight oligomers and high-molecular-weight oligomers including protofibrils, and rapidly change forms and constitute fibrils (Fig. 2). Therefore, obtaining monoclonal antibodies against all Aβ oligomer forms is challenging. Recently, Irie *et al.* analyzed Aβ42 aggregates using solid-state NMR along with systematic proline replacement and revealed that Aβ42 adopts two conformations: one taking a turn at positions 22 and 23 and the other at 25 and 26 [13, 19, 23]. These studies revealed that Aβ42 taking turn at 22/23, named a toxic conformer, is highly aggregative and neurotoxic [13]. The structure has been supposed to be a common form between toxic forms of amyloid oligomers. If we would thus obtain specific antibodies against toxic amyloid oligomer conformers, we could detect all toxic amyloid oligomer forms that display strong neurotoxicity.

At first, Irie *et al.* developed a conformation-restricted Aβ with intramolecular disulfide bonds at positions 17 and 28 (SS-Aβ) to fix the toxic turn at positions 22 and 23 without proline replacement [21]. SS-Aβ does not form fibrils and remains stably in the form of Aβ oligomers. Next, they produced a specific antibody against SS-Aβ42 (TxCo1). ELISA methods revealed that TxCo1 reacts with SS-Aβ42, although the wild-type Aβ42 could not be detected [14]. After obtaining approvals from the Ethics Committees of Shiga University of Medical Science (R2018-101), Kyoto University (G0664-4) and Fukushimura Hospital (403), we examined sections of postmortem human brains from AD patients and control cases by immunohistochemistry using TxCo1 as described previously [1, 14]. Briefly, we deparaffinized the paraffin sections in xylene for 5 min thrice, then rehydrated in 100%, 90%, and 70% EtOH. After washing with tap water, we incubated the samples in 0.3% hydrogen peroxidase solution for 15 min at RT followed by 70% formic acid for 12 min at RT for antigen retrieval. After washing with tap water, we performed antigen retrieval in 1 mM EDTA (pH 8.0) by boiling for 10 min. We then blocked the samples in PBS with 5% normal donkey serum and 0.2% Triton X-100 for 1 hr at RT. Next, we incubated the samples with the TxCo1 antibody at 1 μg/ml in the blocking buffer for 16 hr at 4°C. Finally, we applied the ABC methods and DAB reactions for colorization.

Although the TxCo1 antibody did not stain any structures in the control (Fig. 3A), it revealed multiple senile plaque-like depositions in the brain tissues of AD patients (Fig. 3B). The immune-absorption test demonstrated that SS-Aβ42, but not wild-type Aβ42, abolished the staining in the AD-affected brain (Fig. 3C and D). The double immunostaining using TxCo1 and the antibody against Aβ peptides (mOC64; ab201060: Abcam, Cambridge, MA, USA) demonstrated that some TxCo1-positive structures colocalized with mOC64-immunoreactiitiy, and some TxCo1-positive structures were not stained by mOC64 (Fig. 4). These results indicate that some Aβ structures stained with TxCo-1 antibody are different from Aβ plaques observed by using other Aβ antibodies [14]. Further studies will be needed to clarify the molecules recognized by TxCo1 antibody.

IV. Imaging Mass Spectrometry Using Chemicals That Bind Specifically to Aβ Oligomers

Fluorescent compounds such as thioflavin-T and Congo Red that bind specifically to Alzheimer's lesions

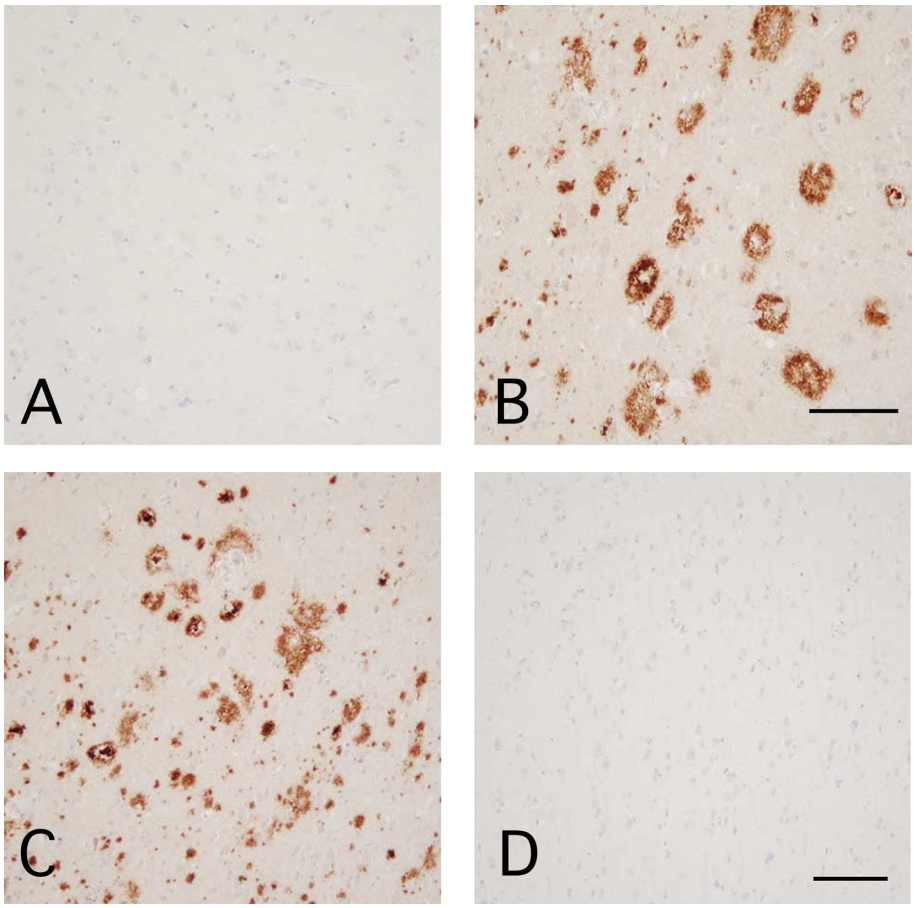

Fig. 3. (**A** and **B**) Immunohistochemistry using TxCo1 in control (**A**) and AD-affected brain samples (**B**). Bar = 100 μm. **C** and **D**: Immuno-absorption test using wild-type (**C**) or SS-Aβ (**D**) peptides. Wild-type Aβ peptides could not reduce the staining intensity while SS-Aβ peptides abolished the signal. Bar = 200 μm.

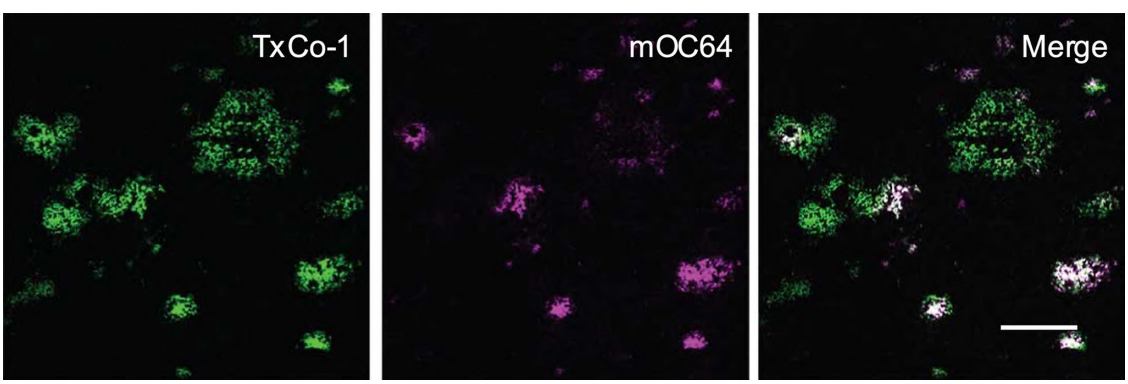

Fig. 4. Double immunofluorescent histochemistry using TxCo1, Aβ antibody (mOC64) and the merged image. Bar = 50 μm.

such as senile plaques and neurofibrillary tangles have been used for histochemical staining. These low-molecular-weight compounds generally have lower sensitivity and specificity than immunohistochemical methods. However, these compounds have been actively studied recently as positron emission tomography and magnetic resonance imaging (MRI) diagnostic probe candidates [26, 29]. Nevertheless, only a few studies report on low-molecular-weight compounds that would specifically bind Aβ oligomers.

Shiga-Y compounds are based on curcumin, a natural food component, and are relatively safe. Curcumin inhibited Aβ aggregation and fibril formation and reduced amyloid pathology in a transgenic mouse model for AD [9]. Significant color change could be observed upon aggregate addition into an F-methyl-curcumin (Shiga-Y5)-containing

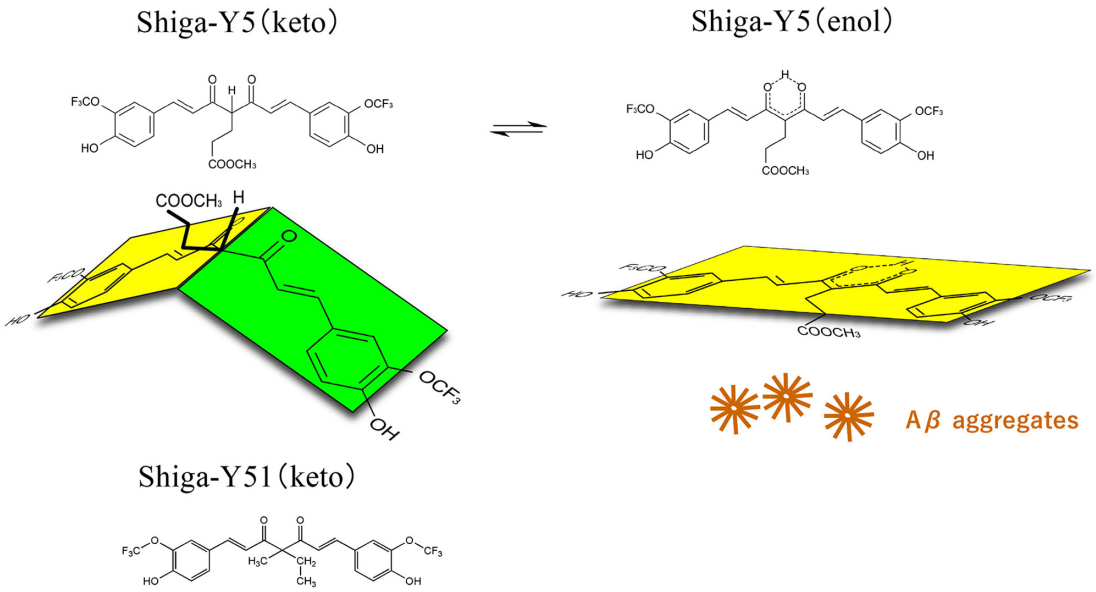

Fig. 5. Shiga-Y5 and Shiga-Y51 keto and enol tautomerisms. In the case of Shiga-Y5, both the enol and keto forms could be observed.

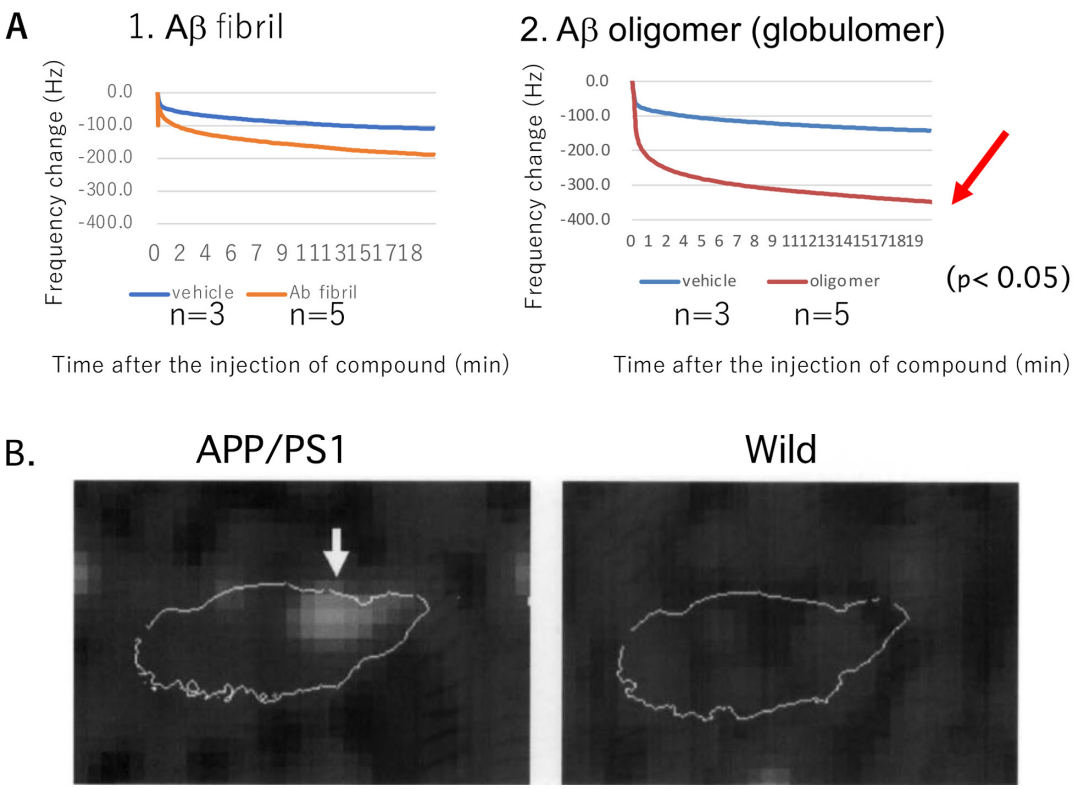

Fig. 6. QCM analysis (**A**) and fluorine-MRI imaging using 7-tesla MRI (**B**). (**A**) The QCM analysis indicates significantly reduced frequency upon Aβ oligomer (globulomer) addition (red arrow; $p < 0.05$). (**B**) ^{19}F signals detected in the brain region of APP/PS1 mice (white arrow).

solution, while no such change occurred when the Shiga-Y5 solution was supplemented with Aβ monomers [32]. Since curcumin and its derivatives exist in equilibrium between keto and enol tautomers, we assume that the structural conversion of curcumin derivatives could occur during Aβ aggregate binding leading to the color change (Fig. 5). Our NMR analysis revealed that Shiga-Y5 exists predominantly in the keto form in the aqueous buffer (pH 7.5) and Shiga-Y5 undergoes enolization upon Aβ aggregate binding. Detailed study of the binding mode of enol type with amyloid fibrils revealed that enol type of Shiga-Y compound binds to Aβ aggregates [32]. In addition, when

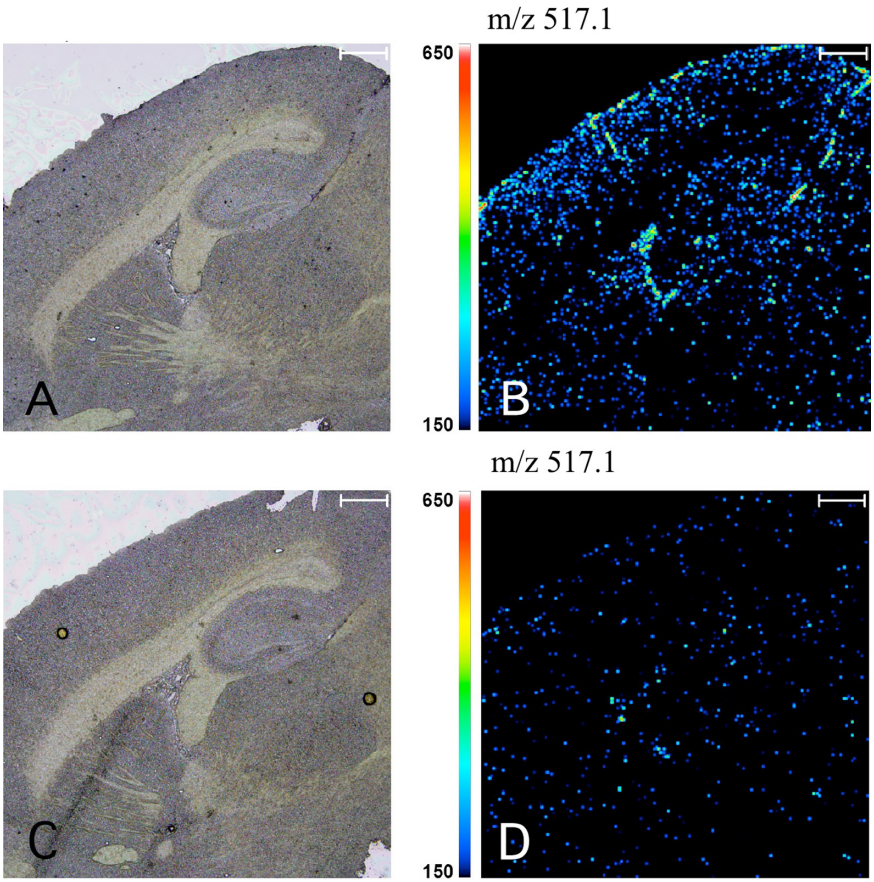

Fig. 7. Optical images (**A** and **C**) and imaging mass spectrometry (**B** and **D**) in the brain samples of APP/PS1 (**A** and **B**) and wild-type (**C** and **D**) mice. Shiga-Y51 signals were detected in the brain of APP/PS1 mice (**B**) but could not be observed in wild-type animals (**D**). Bars = 600 μm.

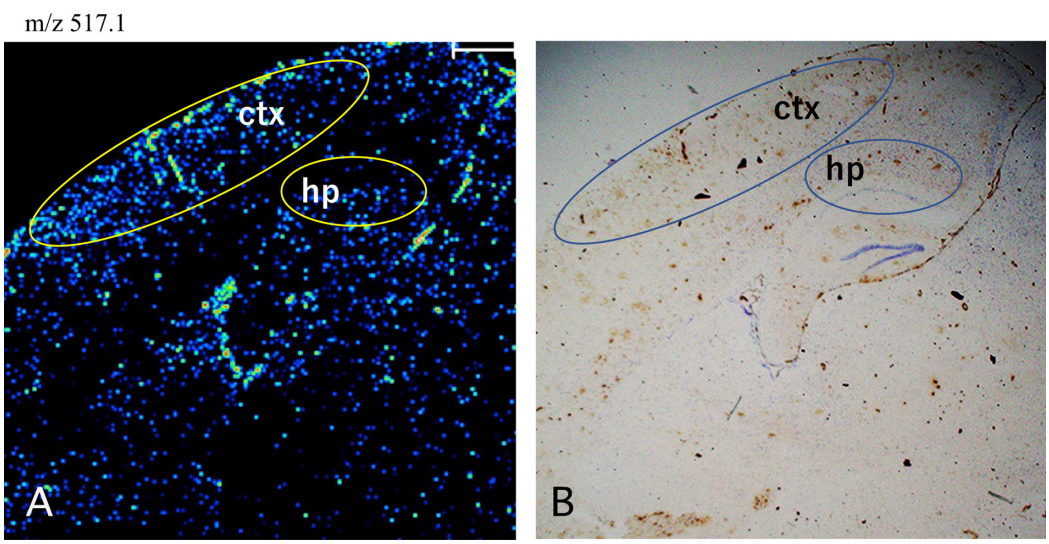

Fig. 8. Comparison of imaging mass spectrometry (**A**) and immunohistochemistry (**B**) using the antibody against toxic conformers of Aβ oligomers (11A1). Ctx and hp indicate the cerebral cortex and the CA1 region of the hippocampus, respectively. Bar = 600 μm.

the structure changed in a way that it could only take the keto form, no Aβ aggregate binding occurred [32]. These results suggest that the Shiga-Y compound enol and keto forms bind and release senile plaques, respectively (Fig. 5).

We also used a 27-MHz QCM analyzer (Affinix Q; Initium, Tokyo, Japan) in order to monitor interactions

between Shiga-Y5 or thioflavin-T with Aβ fibrils or oligomers (globulomer). Our QCM analysis revealed significant frequency decreases in both globulomer- and Aβ fibril-immobilized electrodes compared with control when Shiga-Y5 was injected into the vessel at a final concentration of 10 μM [33]. In contrast, QCM analysis with thioflavin-T injected at a final concentration of 30 μM indicated a significant frequency decrease in the fibril-immobilized electrode, but no difference between control and the globulomer-immobilized electrode [33]. These results indicate that Shiga-Y5 directly interacts with Aβ oligomers and Aβ fibrils [33].

Thioflavin-T displays a planar structure and binds amyloid fibrils but not Aβ oligomers. However, Shiga-Y5 binds both amyloid fibrils and Aβ oligomers. The enol-form of Shiga-Y5 that binds Aβ fibrils exhibits a planar structure. Therefore, the keto form of Shiga-Y5 suggestibly binds Aβ oligomers. In order to test this hypothesis, we synthesized a curcumin derivative, Shiga-Y51, that only displays the keto form (Fig. 5). Our QCM analysis demonstrated that Shiga-Y51 could bind Aβ oligomers but not Aβ fibrils (Fig. 6A). After obtaining approval from the Animal Care and Use Committee at the Shiga University of Medical Science (Number 2018-2-12), we administered 200 mg/kg Shiga-Y51 to genetically modified mice with AD (APP/PS1) and wild-type animals, imaged them with a 7-Tesla MR scanner (Fig. 6B), then removed the brains and performed immunohistochemistry to compare the Sihga-Y51 distribution in the two backgrounds using 11A1 (haptene: E22P-Aβ9-35), an Aβ oligomer antibody [24]. Unfortunately, Shiga-Y51 is not fluorescent, we thus used imaging mass spectrometry to reveal its distribution. The imaging mass spectrometry revealed Shiga-Y51 signals in the brain samples of APP/PS1 but not wild-type mice (Fig. 7A–D). Finally, we compared the results of imaging mass spectrometry (Fig. 8A) and immunohistochemistry (Fig. 8B) using 11A1. The Shiga-Y51 distribution was consistent with that of Aβ oligomers revealed by the 11A1 immunohistochemistry (Fig. 8). Taken together, these results indicate that Shiga-Y51 is a low-molecular-weight compound that specifically recognizes Aβ oligomers.

V. Conclusion

The history of AD research provides multiple examples for the histological detection of pathological structures, providing clues to understanding disease pathology. Since Aβ oligomers are detected in the early stages of AD and display potent neurotoxicity, they represent AD diagnostic and therapeutic targets. However, Aβ oligomer visualization in tissues is difficult. We attempted to visualize Aβ oligomers in the brain tissues of patients with AD and transgenic mouse models for AD. The TxCo1 immunohistochemistry revealed Aβ oligomer distributions in post-mortem AD-affected human brain samples. The imaging mass spectrometry using Shiga-Y51 presented Aβ oligomer distributions in the brain samples of a transgenic AD mouse model. We are convinced that these two approaches would contribute to elucidating the underlying pathology mechanisms of AD.

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