Detection of Amyloid β Oligomers with RNA Aptamers in AppNL-G/F/NL-G-F Mice: A Model of Arctic Alzheimer’s Disease

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1. INTRODUCTION

Nucleic acid aptamers are molecular recognition tools for variable targets and offer advantages over antibodies with respect to diversity, production costs, and the need for animal experiments, among other reasons. However, difficulties associated with in vitro selection, characterization, and validation have hampered further research into aptamer development. In particular, the design of hapten molecules depends on empirical rules governing several parameters, such as the separation methods and initial template design. Metastable or aggregative molecules are considered among the most difficult molecules to generate aptamers exemplified amyloidogenic proteins such as 40-mer amyloid β-protein (Aβ40), whose accumulation is relevant to the pathology of Alzheimer’s disease (AD), are among the most difficult molecules for aptamer recognition because they are prone to aggregate in heterogeneous forms. In addition to designing haptons for in vitro selection of aptamers, the difficulties involved in determining their effect on Aβ42 oligomerization impede aptamer research. We previously developed three RNA aptamers (E22P-AbD4, -AbD31, and -AbD43) with high affinity for protofibrils (PFs) derived from a toxic Aβ42 dimer. Notably, these aptamers recognized diffuse staining, which likely originated from PFs or higher-order oligomers with curvilinear structures in a knock-in AppNL-G/F mouse, carrying the Arctic mutation that preferentially induced the formation of PFs, in addition to a PS2Tg2576 mouse. To determine which oligomeric sizes were mainly altered by the aptamer, ion mobility mass spectrometry (IM–MS) was carried out. One aptamer, E22P-AbD43, formed adducts with the Aβ42 monomer and dimer, leading to suppression of further oligomerization. These findings support the utility of these aptamers as diagnostics for AD.

Murakami and colleagues recognize protofibrils (PFs), which possess a curvilinear structure. E22P-Aβ42, which resembles the toxic conformer of Aβ42, was identified by Irie and colleagues. E22P-Aβ42 has demonstrated greater neurotoxicity than wild-type Aβ42 as it can form a turn structure at Glu22 and Asp23 (Figure 1A). E22P-V40DAP-Aβ42 dimer (Figure 1A) with a covalent linker at Val40 in the C-terminal hydrophobic core, which plays an important role in oligomer formation, can form PFs following incubation.

One aptamer, E22P-AbD43, delayed the nucleation phase of Aβ42 estimated by the thioflavin-S fluorescence test and suppressed its associated neurotoxicity toward SH-SYSY human neuroblastoma cells. However, there was no information concerning the oligomer size to be targeted by the aptamer. Ion mobility–mass spectrometry (IM–MS) combined with native ionization techniques have enabled us to identify the distribution of the Aβ42 oligomer under nearly native conditions. The present study illustrates the
assessment of antiligomer RNA aptamers by IM−MS. Based on the immunostaining results of the diffuse staining originating from PFs by E22P-AbD43 in a transgenic mouse model of AD (PS2Tg2576), further validation of the RNA aptamers was performed using not only PS2Tg2576 but also an APP knock-in AppNL-GF/NL-GF mouse model developed by Saido and colleagues. AppNL-GF/NL-GF mice possess the Arctic mutation that is prone to induce PF formation from Aβ.29,30

2. RESULTS AND DISCUSSION

2.1. Analysis of RNA Aptamer Sequence. Based on previous CD and FT-IR studies of E22P-AbD43, the formation of a G-quadruplex structure may influence their binding to PFs. The three aptamers (E22P-AbD4, -AbD31, and -AbD43) showed a slightly higher guanine content than the average (25%) (Figure 1B). Consecutive guanine residues in the G-quadruplex are held in a square G-tetrad planar via Hoogsteen hydrogen bonding.31 The G-score deduced from quadruplex-forming G-rich sequences (QGRS) Mapper32 supported the possible existence of the G-quadruplex in E22P-AbD4 (highest G-score = 17) and E22P-AbD31 (highest G-score = 16) (Figure 2A). These values of E22P-AbD4 and -AbD31 are nearly comparable to SPB133 (highest G-score = 21) and TBA34 (highest G-score = 20) as typical examples of DNA oligonucleotide forming G-quadruplex (Figure 2B). These indicate the preferable formation of the G-quadruplex in E22P-AbD4 and E22P-AbD31. Further analysis of E22P-AbD43 for the potential formation of G-quadruplex such as NMR will be needed because of unsuccessful calculation of the G-score (data not shown).

2.2. RNA Aptamers Recognize PFs in AD Models of APP Transgenic Mouse and APP Knock-In Mouse. In addition to E22P-AbD43 observed in the previous study, we newly performed histochemical analysis for E22P-AbD4 and E22P-AbD31 using a transgenic mouse model for AD (PS2Tg2576), carrying the human wild-type Aβ sequence.34 Both E22P-AbD4 and E22P-AbD31 recognized diffuse staining of Aβ mainly in the cerebral cortex and hippocampus regions (Figure 3A) like E22P-AbD43.11 The immunoreactivity of E22P-AbD31 was slightly stronger than that of E22P-AbD4. By counting the numbers of aggregates, we found that the diffuse staining in both the cerebral cortex and hippocampus were exclusively recognized by both the aptamers. As shown in Figure S1A (Supporting Information), senile plaques were observed in PS2Tg2576 by an anti-Aβ-N-terminus antibody (82E1)35 similarly to the previous reports.11,34 The diffuse staining, which was less fuzzy compared with "diffuse plaques", detected by these aptamers may have originated from PFs or higher-order oligomers with curvilinear structures derived from Aβ. To further verify reactivity toward Aβ oligomers by the aptamers, a 4-month-old knock-in mouse (AppNL-GF/NL-GF) that harbors the APP-related Swedish and Beyreuther/Iberian mutations with the Arctic mutation within the Aβ gene sequence was used. Saido and colleagues reported that 4-month-old AppNL-GF/NL-GF mice exhibited subcortical amyloidosis28 and that these phenotypes are consistent with the pathology of human Arctic mutation carriers.37 As shown in Figure 4A, the diffuse staining was immunostained by all of the aptamers including E22P-AbD43 (Figure 4B). The shape of the diffuse staining appeared to be similar to observations of PS2Tg2576
In contrast, there were almost no aggregates with a dense core probed by 82E1 in AppNL-β-F/NL-β-F (Figure S1B). This result does not largely contradict the report by Latif-Hernandez et al., who reported minor staining by the 6E10 monoclonal antibody specific for Aβ1-16. 82E1 reacted with diffuse staining in AppNL-β-F/NL-β-F like the aptamers, possibly because the N-termini of the PFs were exposed.

2.3. RNA Aptamer Formed Adducts with Aβ42 Oligomer as Determined by IM-MS Analysis. To characterize the early oligomeric profile of Aβ42 by RNA aptamers, ion mobility–mass spectrometry (IM–MS) was carried out. Avoiding the disruption of noncovalent interactions among Aβ oligomers by not using organic solvents enabled us to observe the near-native status of Aβ oligomers in the presence of aggregation inhibitors.

After deconvolution based on the observed mass, peaks corresponding to oligomeric orders of Aβ42 and Aβ42-RNA adducts were assigned to the series of multivalent ions depending on their drift time (Table S1). n denotes an integer corresponding to the number of units coexisting in the solution \( n = 1, 2, 3, \ldots \) denotes monomer (Mon), dimer (Dim), trimer (Tri), …, respectively. The Aβ42 dimer and trimer peaks were apparently found after dissolution with the buffer (Figure 5A). These adducts were not detectable after incubation for 1 h at 37 °C due to further aggregation of Aβ42 (data not shown). In contrast, the addition of E22P-AbD43, whose binding ability to PFs was the strongest among the three aptamers, induced the disappearance of these oligomer peaks, and the corresponding adduct peaks of the monomer and dimer with RNA were observed (Figure 5B), meaning that the formation of dimers, which are fundamental subunits of Aβ42 oligomers \( n \geq 3 \), was suppressed.

3. CONCLUSIONS

To the best of our knowledge, we are the first to demonstrate the accumulation of diffuse staining deduced from PFs in AppNL-β-F/NL-β-F mouse by anti-Aβ42 oligomer RNA aptamers and that in particular E22P-AbD43 prevented further
oligomerization of Aβ42 by directly interfering with the monomer or dimer unit using IM−MS. This interference may be associated with the formation of the G-quadruplex. Further studies will be required to clarify the structural information of RNA for its specific binding to the target and further characterization of affinities to other amyloidogenic proteins.

E22G-Aβ4230 and E22G-Aβ4029 preferably formed PFs in vitro. The levels of PFs reportedly correlated with the impairment of spatial learning in Arctic AD transgenic mice.40 The animal study on Arctic-mutant APP Tg lines suggested that the PF-related oligomeric species (>30-mer) could contain Aβ56 (ca. 12-mer) as a nonfibrillar Aβ assembly.41 These findings suggest that diffuse staining detected by the aptamers could contain PFs. The age of 4 months in AppNL-G-F/NL-G-F may correspond to the initiation of subtle disease-related behavioral changes.38 Clinical studies have suggested that the accumulation of PFs is a promising biomarker of even mild cognitive impairment before the onset of AD.42 The humanized monoclonal antibody (BAN2401) recognizing PFs is also a promising drug candidate according to a phase 2 randomized trial.43 Recently, the involvement of neuronal membrane damage in the neurotoxicity induced from high-order oligomers of Aβ42 such as PFs was reported.44 Further experiments to shorten the length and to incorporate the modified base into the RNA aptamers for their passage

Figure 3. Histochemical analysis of PS2Tg2576 mouse brains using RNA aptamers. (A) Representative micrographs were obtained after treatment with E22P-AbD4 and -AbD31 (400 nM). High-magnification images (scale bar = 50 μm) of the area (scale bar = 500 μm) inside the rectangles of the hippocampus are shown within each picture. Arrowheads indicate diffuse staining. (B) Comparison of the numbers of diffuse staining and senile plaques stained in three parts in the cortex and hippocampus of (A), respectively.

Figure 4. Histochemical analysis of AppNL-G-F/NL-G-F mouse brains using RNA aptamers. (A) Representative micrographs were obtained after treatment with E22P-AbD4, -AbD31, and -AbD43 (400 nM). High-magnification images (scale bar = 50 μm) of the area (scale bar = 500 μm) inside the rectangles of the hippocampus are shown within each picture. Arrowheads indicate diffuse staining. (B) Comparison of the numbers of diffuse staining and senile plaques stained in three parts in the cortex and hippocampus of (A), respectively.
across the blood–brain barrier toward Aβ imaging application are currently underway.

4. MATERIALS AND METHODS

4.1. Preparation of RNA Aptamers. The aptamers were obtained using an in vitro selection method known as the systematic evolution of ligands by exponential enrichment (SELEX) using a membrane filter methodology, as reported previously.11 Using the ssDNA (Eurofins; Tokyo, Japan) of the RNA aptamer, RiboMAX Large-Scale RNA Production System-T7 (Promega, Madison, WI) was used to generate the RNA transcript. After phenol–chloroform extraction and desalting using Illustra MicroSpin G-25 columns (GE Healthcare), the integrity of RNA was confirmed by electrophoresis using 6% Tris-borate-EDTA gels (Invitrogen) and stained by SYBR Green (TaKaRa). RNA quantification was performed using a Web server (http://bioinformatics.ramapo.edu/QGRS/index.php). This is a scoring system that calculates the probability of forming a stable G-quadruplex. The instrument was operated in negative ion mode with a capillary voltage of 1.0 kV, a sample cone voltage of 40 V. Data acquisition and processing were performed with the MassLynx (V4.1) and DriftScope (V2.8) software supplied with the instrument. The Csl cluster ions were used for the m/z scale as a calibrator.

4.2. QRGS Mapping. Prediction of putative quadruplex-forming G-rich sequences (QRGS) in nucleotide sequences was performed using a Web server (http://bioinformatics.ramapo.edu/QGRS/index.php). This is a scoring system that calculates the probability of forming a stable G-quadruplex. The putative G-quadruplexes are identified using the following motif: G

4.3. Histochemical Staining. All experimental procedures were performed, as previously described,11 in accordance with specified guidelines for the care and use of laboratory animals and were approved by the Animal Care and Use Committee of Chiba University.

Five micrometer thick coronal paraffin-embedded sections were prepared from 4% paraformaldehyde-fixed brain hemispheres of 6-month-old PS2Tg2576 mice10 and 4-month-old AppNL-G-F/NL-G-F knock-in mice.28 After deparaffinization and hydration, the slices were autoclaved at 120 °C for 20 min to allow antigen activation. To inactivate the endogenous peroxidase, brain sections were soaked in methanol with 0.1% H2O2, at room temperature for 30 min. After washing with ice-cold phosphate-buffered saline (PBS) plus potassium (PBS-K; 10 mM sodium phosphate, 140 mM NaCl, pH 7.4) containing 0.02% Tween-20 (PBS-T), blocking was performed in a blocking buffer, PBS-T with 10 μg/mL bovine serum albumin (Nacalai, Kyoto, Japan) and 10 μg/mL yeast tRNA (Nacalai) at room temperature for 60 min. The biotinylated aptamer (400 nM) diluted in RNase-free water (Invitrogen, Carlsbad, CA) with 1 mM EDTA was added at room temperature for 60 min, followed by reaction with horseradish peroxidase (HRP)-conjugated avidin by the VECTASTAIN ABC HRP Kit (Vector Laboratories, Burlingame, CA) for 30 min at room temperature. Alternatively, the sections were treated with 82E1 (1 μg/mL) and diluted with PBS-T at room temperature for 60 min, followed by reaction with the biotinylated secondary antibody for 30 min at room temperature before incubation with HRP-conjugated avidin by the VECTASTAIN ABC HRP Kit (Vector) for 30 min at room temperature. To visualize the signals, brain sections were treated with 3′,5′-diaminobenzidine (Dojindo, Kumamoto, Japan) at 37 °C for 12 h (RNA aptamer) or at room temperature for 12 min (82E1). Nuclei were stained with hematoxylin reagent (Wako). Brain sections were mounted with Permount (PALMA, Tokyo, Japan) after dehydration and soaking in xylene.

4.4. Ion Mobility–Mass Spectrometry (IM–MS). Aβ42 was dissolved in 0.1% NH4OH to a concentration of 400 μM and RNA was dissolved in nuclelease-free water (Promega, Madison, WI) to a concentration of 400 μM. Next, the Aβ42 and RNA solutions were diluted 10-fold and 5-fold, respectively, in 25 mM ammonium acetate (pH 7.4). The resulting solution (40 μM Aβ42, 80 μM RNA) was centrifuged for 4 min at 2000 g (4 °C) before infusion into an MS apparatus using a glass capillary (Nanoflow Probe Tip, Waters). Mass spectra and ion mobility experiments were accomplished on a SYNAPT G2-Si HDMS (Waters) using a nanoelectrospray as an ionization source, as reported previously.39 The instrument was operated in negative ion mode with a capillary voltage of 1.0 kV, a sample cone voltage of 10 V, and a source temperature of 50 °C. For the ion mobility measurement, nitrogen gas was used in the ion mobility cell, and the cell pressure was maintained at approximately 2.95 mbar with a wave velocity of 300–1000 m/s and a wave height of 10–40 V. Data acquisition and processing were performed with the MassLynx (V4.1) and DriftScope (V2.8) software supplied with the instrument. The Csl cluster ions were used for the m/z scale as a calibrator.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.0c02134.
Representative micrograph of brain histochemical staining of the PS2Tg2576 mouse and the AppNL-G-F/NL-G-F mouse using anti-N-terminus of the Aβ (82E1) antibody (Figure S1). The list of calculated and observed masses of Aβ42 and Aβ42 treated with E22P-AbD43 in MS–MS measurements (Table S1) (PDF)

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**Notes**

The authors declare no competing financial interest.

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