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Research Paper biology

Classification of monoclonal antibodies to measure the progress of $A\beta_{1-42}$ aggregation

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ABSTRACT

Amyloid beta protein1-42 ($A\beta_{1-42}$) accumulates in the brains of Alzheimer's disease (AD) patients. Soluble amorphous aggregates were prepared by dissolving $A\beta_{1-42}$ in 1,1,1,3,3,3-hexafluoro-2-propanol. Their sizes ranged from 20 to 400 nm on their major axes, as measured by atomic force microscopy. The aggregates were used for immunization to produce nine IgG monoclonal antibodies. All the antibodies reacted with the aggregates of more than 300 kDa and exhibited decreased reactivity against aggregates more than 0.22 µm. These antibodies showed little activity against fibrils, and they showed relatively low reactivity against the monomer and the large oval aggregate (LOA) that was prepared in the presence of 2.2 mM $A\beta_{16-20}$ to suppress the fibril formation. Centrifugation of the aggregates decreased the reactivity of antibodies, suggesting that the epitopes were unstable. These antibodies will be useful for AD investigators since they allow for the detection of different sizes and shapes of $A\beta_{1-42}$ aggregates.

KEYWORDS : Alzheimer's disease, Aβ1-42, soluble amorphous aggregates, monoclonal antibody, epitope

INTRODUCTION

Amyloid beta protein (Aβ) is found in the brain plaques of Alzheimer's disease (AD) patients.[1]Aβ consists of 40 ($A\beta_{1-40}$) and 42 ($A\beta_{1-42}$) amino acid residues and is produced by hydrolytic digestion of the amyloid protein precursor by β- and γ-secretase.[2] $A\beta_{1-42}$ is more toxic than $A\beta_{1-40}$ due to its more rapid aggregation rate, despite the fact that $A\beta_{1-42}$ protein levels in the brain are approximately 10 times lower than those of $A\beta_{1-40}$.[3]

There are two forms of $A\beta_{1-42}$ aggregates, the fibril and amorphous forms.[4] Prior to fibrillization, Aβ forms a spherical intermediate that is 15–35 nm in diameter and consists predominantly of a β-sheet structure.[5] The Aβsoluble aggregates are more toxic than the fibril and monomeric forms.[2] Aggregate toxicity is probably derived from the interaction of aggregates with the membrane,[6] membrane receptors,[7] and DNA,[8] the formation of metal-Aβ complexes that cause oxidative stress,[9] and the membrane pore, which influences ionic homeostasis.[10,11] Aggregate toxicity is, in part, dependent on aggregate size; this is likely due to the distinct tertiary structures on the Aβ surface.[12] However, tertiary structures on the Aβ surface are unstable, and their detection is difficult, especially in vivo.

The pentapeptide ($A\beta_{16-20}$) consists of Aβ amino acids 16–20 and binds to the β-sheet structure of aggregate intermediates to prevent fibril formation and to induce the formation of amorphous aggregates.[13] In our previous work, we prepared a large oval aggregate (LOA) of $A\beta_{1-42}$ that had an average size of 370 and 220 nm on the major and minor axes, respectively.[14,15] LOA are produced by incubating 0.22 mM (1.0 mg/mL) $A\beta_{1-42}$ for 16 h at 37°C in the presence of 2.2 mM $A\beta_{16-20}$ with slow rotation. Large aggregate LOA are purified by centrifugation-filtration using a 0.22 µm filter.

The larger aggregates and fibrils in the extracellular spaces of the brain of an AD patient are important for clinical diagnosis.[16] Various types of monoclonal antibodies against Aβ have been used to investigate the aggregate form of Aβ.[17] Some monoclonal antibodies are capable of distinguishing between aggregates based on size and fibril formation.[18] Monoclonal antibodies targeting specific 16.5–25 kDa amyloid oligomers,[19] 16.5–25 kDa amylospheroids (ASPDs, 10–15 nm in diameter), and 4 kDa to more than 250 kDa fibrillar oligomers[20] have previously been reported. These antibodies recognize an epitope formed on the surface of the assembled Aβ. We previously obtained monoclonal antibody 31-2 against LOAs and suggested that it targets a specific Aβ surface epitope.[14]

In this study, soluble $A\beta_{1-42}$ aggregates were prepared and monoclonal antibodies against the aggregates were generated. These antibodies strongly reacted with aggregates >300 kDa and sizes <0.22 µm, and showed decreased reactivity against aggregates >0.22 µm and LOA. These data suggest the antibodies generated here target unique epitopes on the surface of aggregates >300 kDa and, furthermore, are a useful tool for elucidating the Aβ aggregation process.

MATERIALS AND METHODS

Preparation of aggregates and monomers

The $A\beta_{1-42}$ was purchased from AnyGen Co. Ltd. Korea To prepare soluble amorphous aggregates exhibiting high aqueous solubility, 0.11 mM $A\beta_{1-42}$ was dissolved in 1,1,1,3,3,3-hexafluoro-2-propanol and incubated at 4°C for 16 h followed by 37°C for 3 h, at which point the solution was lyophilized. These steps, including dissolution and lyophilization, were repeated twice. The soluble aggregates were prepared from lyophilized $A\beta_{1-42}$ in deionized water. For monomer preparation, 0.22 μM of the lyophilized $A\beta_{1-42}$ was dissolved in 1,1,1,3,3,3-hexafluoro-2-propanol; the solution was incubated for 16 h at 4°C and then sonicated at 38 kHz (US-18KS, SND Co. Ltd. USA.) for 16 h at room temperature. Aggregate $A\beta_{1-42}$ was prepared by incubation of 0.22 mM $A\beta_{1-42}$ in the presence of 2.2 mM $A\beta_{16-20}$ in Dulbecco's phosphate-buffered saline without Ca^{2+} or Mg^{2+} (PBS, Wako Japan) with rotation at 7 rpm for 16 h at 37°C. LOA was purified by filtration with 0.22-μm diameter filters to remove small aggregates.[14] Assembled Aβ was separated by size using Ultracel Regenerated cellulose centrifugal filters (Merck Millipore USA) or polyethersulfone filters with 300 kDa cut-offs (Vivaspin; Sartorius USA) at 100 × *g* for 5 min at 4°C. The concentration was determined by absorbance at 280 nm with the molar coefficients of tyrosine assumed to be 1,450 M^{-1} cm^{-1}.[21] To prepare fibrils, 0.22 mM $A\beta_{1-42}$ in deionized water was incubated at 37°C for 16 h.

Atomic force microscopy

The shapes and sizes of these assemblies were observed by atomic force microscopy (AFM, JSPM-5200; JEOL Ltd. Japan). The $A\beta_{1-42}$ solution (10 μL) was dropped on fresh mica and dried by desiccation. Measurements were performed using the altering current (AC) mode at room temperature and a frequency of about 190 kHz, typical of resonances with a 4.5 N/m spring constant.

Monoclonal antibody preparation

Monoclonal antibodies against the soluble aggregates were made from mice immunized with soluble aggregates. Their splenocytes were fused with P3U1 myeloma cells as previously described.[14] Hybridomas producing monoclonal antibodies were isolated by limiting dilutions. The cells were cultured in RPMI 1640 medium (Invitrogen USA) with HAT supplement (Invitrogen USA) under 5% CO_2 for 10 days. The hybridoma cells, which secrete the monoclonal antibody that reacts with the soluble aggregates, were screened by ELISA (data not shown). The antibody sub-class was analyzed with the IsoStrip Kit (Roche USA), and IgG sub-classes were selected because of their higher stability and reactivity. The antibodies were purified by 50% ammonium sulfate precipitation followed by protein an affinity chromatography (GE Healthcare USA).

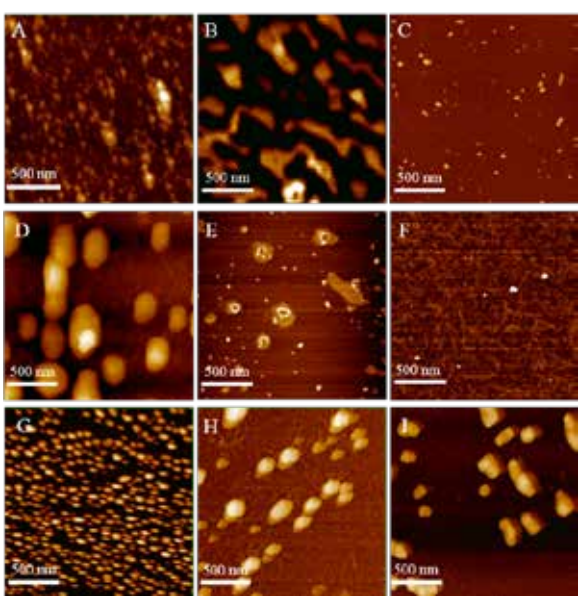

Figure 1. AFM images of the aggregate assemblies. The soluble aggregates (A) were prepared by dissolving in 1,1,1,3,3,3-hexafluoro-2-propanol. The soluble aggregates remained amorphous after 16 h (B) and 32 h (C).$A\beta_{1-42}$ in deionized water (D) was incubated for 8 h (E) and 16 h to form fibril (F). $A\beta_{1-42}$ in deionized water in the presence of $A\beta_{16-20}$ was incubated for 8 h (G) and 16 h (H). The soluble aggregates were centrifuged at 14,000 rpm for 10 min (I).

Enzyme-linked immunosorbent assay (ELISA)

ELISA was performed as previously described.[14] Briefly, ELISA plates (F96 MXISORP NUNC-IMMUNO PLATE, Thermo Fisher Scientific Inc. USA) were coated with 100 μL of 1 μg/mL Aβ monomer or the assemblies in PBS for 16 h at 4°C. For the size-fractionated aggregates, plates were coated with 100 μL of 1 μg/mL. The plates were washed with PBS containing 0.05% Tween 20 and blocked with Immunoblock (DS Pharma Biomedical Japan) for 2 h at 37°C. For the primary antibody and secondary antibody (horseradish peroxidase-conjugated anti-mouse IgG, Sigma-Aldrich USA) application 0.2 μg/well, and SIGMAFAST OPD (Sigma-Aldrich USA) were used according to the manufacturer's instructions. All data are shown with the mean value of at least three measurements and error bars indicate one standard deviation.

RESULTS AND DISCUSSION

Preparation of aggregates

Surface shapes of assemblies were analyzed with an atomic force microscope (AFM). Fig. 1A shows images of the soluble amorphous aggregates of $A\beta_{1-42}$. The soluble aggregates included various aggregate sizes. The average size of the majority of aggregates less than 100 nm was 45 ± 16 nm; however, some aggregates are more than 300 nm on each of their major axes. The soluble aggregates remained amorphous after 16 h (Fig. 1B) and 32 h (Fig. 1C) of incubation. $A\beta_{1-42}$ dissolved in deionized water formed large amorphous aggregates (Fig. 1D). After 8 h of incubation with the $A\beta_{1-42}$ aggregates dissolved in deionized water, most of the aggregates were transformed (Fig. 1E), followed by fibril formation after 16 h of incubation (Fig. 1F). In the presence of excess $A\beta_{16-20}$ (2.2 mM), the $A\beta_{1-42}$ aggregates dissolved in deionized water were transformed into the amorphous form after 8 h of incubation (Fig. 1G). After 16 h of incubation, the amorphous aggregates formed into LOA (Fig. 1H). The monomer was undistinguishable by AFM.[14]

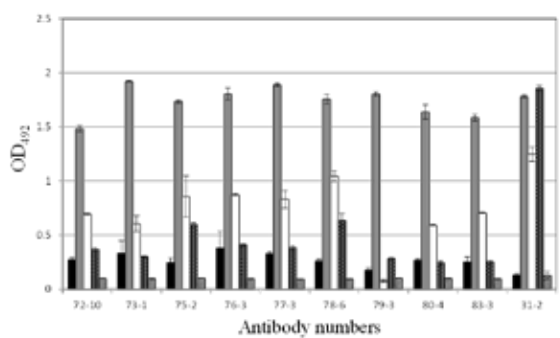

Figure 2. Reactivity of monoclonal antibodies. Reactivity of the nine obtained antibodies and antibody 31-2 against the monomer (■), soluble aggregates (▨), aggregate Aβ1-42 (□), LOA (■), and fibril (▨) were assayed by ELISA.

Antibody specificity

The reactivities of the nine obtained antibodies were analyzed against the soluble aggregates, fibrils, monomers, and LOA by ELISA. Fig. 2 shows the reactivity of the antibodies designated 72-10, 73-1, 75-2, 76-3, 77-3, 78-6, 79-3, 80-4, and 83-3 monoclonal antibodies. All antibodies reacted with the soluble aggregates, but showed little reactivity against the monomeric forms and no significant reactivity against fibrils. Except for 79-3, monoclonal antibodies reacted with not only soluble aggregates, but also with insoluble aggregate $A\beta_{1-42}$. Monoclonal antibody 79-3 showed low reactivity against aggregate $A\beta_{1-42}$. Therefore, monoclonal antibody 79-3 is specific for soluble aggregates. The reactivity of antibody 31-2 against LOA is shown in Fig. 2. It reacted with both soluble aggregates and aggregate $A\beta_{1-42}$. With regard to the reactivity against aggregate $A\beta_{1-42}$, it is reported that 31-2 is specific to LOA of more than 0.22 μm after preparation by filtration.[14])The reason for the reaction of antibody 31-2 against soluble aggregates was evaluated. The sizes of the soluble aggregates varied

from 20 nm to 400 nm on their major axes (Fig. 1). It is thought that monoclonal antibody 31-2 reacts to soluble aggregates $A\beta_{1-42}$ greater than 220 nm in size that were prepared from lyophilized $A\beta_{1-42}$ in deionized water. The soluble aggregates were separated with 0.22 µm, 300 kDa, and 100 kDa filters by centrifugal filtration to investigate the aggregate sizes with which the antibodies reacted. The nine obtained antibodies recognized unique epitopes that are distinct from those recognized by antibody 31-2. We examined the reactivities of these antibodies and antibody 31-2 against aggregates size filtered by ELISA. Fig. 3 shows the reactivity of the antibodies. against the size-separated aggregates. The antibodies, except for monoclonal antibody 31-2, showed high reactivity to aggregates that passed through the 0.22 µm filters but that were retained on the 300 kDa filters. The antibodies showed low reactivity against the aggregates that were retained on 0.22 µm filters, and showed no reactivity against aggregates less than 300 kDa. This result suggests that there are unique epitopes on the surface of soluble aggregates that are greater than 300 kDa. On the other hand, monoclonal antibody 31-2 reacts with aggregates larger than 0.22 µm. The result indicates that 31-2 reacts against large, soluble aggregates prepared from lyophilized $A\beta_{1-42}$ in deionized water, and that this reactivity was specific to large aggregates. It is conceivable that 31-2 reacts to LOA in soluble aggregates that have been prepared from lyophilized $A\beta_{1-42}$ in deionized water.

Among large soluble aggregates, those less than 0.22 µm should have more epitopes than the larger aggregates. The epitopes could be generated by a spatial arrangement of $A\beta_{1-42}$ on the surface of the soluble aggregates. The antibodies generated in this study can be used to distinguish between large aggregates of $A\beta_{1-42}$. Since soluble Aβ aggregates mainly exist at 80–500 kDa *in vivo*[22] and Aβ deposits in the brains of AD patients mainly have diameters of 20–40 µm,[23] large aggregates should be distinguishable in investigations of the aggregation process. Antibody 79-3 will contribute to further research by making it possible to determine the localization and size of aggregations *in vivo*, especially they were when they were along with other size-specific antibodies, such as anti-LOA 31-2.

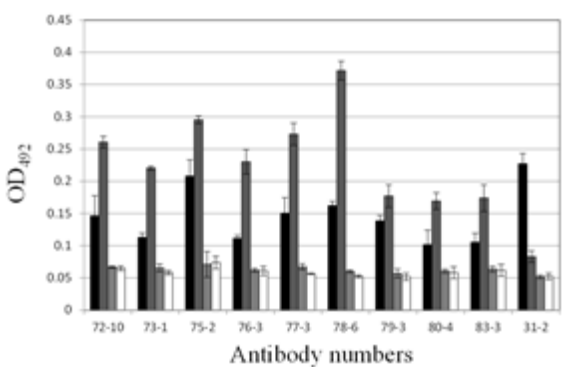

Figure 3. Reactivity against the soluble aggregates separated by size with centrifugal filter units. Reactivity against aggregates that were retained on the 0.22 um (■), 300 kDa (▩), and 100 kDa (▨) filters and filtrate from 100 kDa filters (□) are shown. Effect of centrifugation on the aggregates.

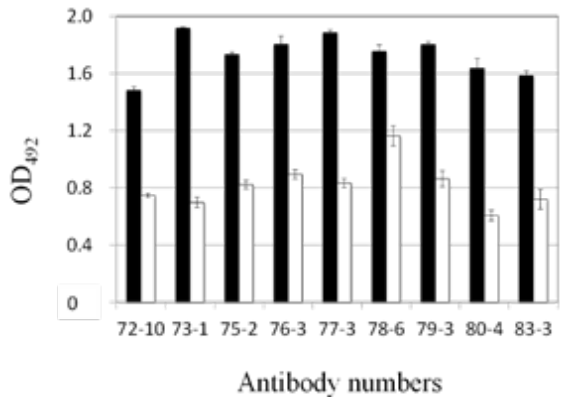

Figure 4. Effect of centrifugation on the surface epitopes of the soluble aggregates. Reactivity against the soluble aggregates before (■) and after centrifugation at 14,000 rpm for 10 min (□) was assessed by ELISA.

The reactivity of the antibodies against the soluble aggregates decreased after centrifugal filtration (Fig. 4). As shown in Fig. 2, the optical density (OD) of soluble aggregates is more than 1.4, which is much higher than that (< 0.4) of the centrifugally separated aggregates shown in Fig. 3. This is probably because the epitopes were degraded by the centrifugal force. Fig. 1 shows an AFM image of the soluble aggregates after centrifugation, and it should be noted that the aggregates are much larger after this treatment than before. These results suggest that centrifugal treatment accelerates aggregation, thus degrading the epitopes

CONCLUSION

The soluble amorphous $A\beta_{1-42}$ aggregates were prepared for immunization to produce nine IgG monoclonal antibodies. All generated antibodies reacted with soluble amorphous aggregates greater than 300 kDa. These antibodies showed relatively low reactivity against LOA and identifying size of $A\beta_{1-42}$ aggregations. Moreover, the reactivities of these antibodies against soluble aggregates decreased after solubilization of the aggregates by centrifugation. These results suggest that unique epitopes exist on the surface of soluble aggregates greater than 300 kDa. These antibodies will be useful for AD research, as they allow for the identification of difference in sized and shapes of $A\beta_{1-42}$ aggregates.

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