Journal of Cellular Biochemistry 99:878–889 (2006)

Amyloid β Oligomerization Is Induced by Brain Lipid Rafts

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Abstract Detergent-resistant lipid rafts are required for the generation of Aβ as they concentrate not only amyloid precursor protein (APP), but also the β- and γ-secretase that convert APP to Aβ. Recently, Aβ has been shown to be oligomerized, which results in neuronal cytotoxicity and synaptic failure. In this study, we have demonstrated that Aβ oligomers appeared immediately after the incubation of Aβ with lipid rafts isolated from the brain tissues of rats, and were converted into few Aβ fibrils, even after longer periods of incubation. The oligomerization of Aβ was not abolished after the brain lipid rafts were treated with heat, or with protease K, implying that the lipid raft proteins were determined not to be prerequisites for Aβ oligomerization. The cholesterol present in the lipid rafts might not be essential to Aβ oligomerization because Aβ oligomerization was not prevented after the cholesterol was removed from the lipid rafts with methyl-β-cyclodextrin (MβCD). The Aβ oligomerization was accelerated by the application of lipid rafts isolated from ganglioside-rich cells, C_2C_{12} cells, whereas this was not observed with the lipid rafts isolated from ganglioside-poor cells SK-N-MC and HeLa cells. In addition, lipid raft-induced Aβ oligomerization was shown to be inhibited in CHO-K1 cells which were defective with regard to ganglioside biosynthesis. This indicates that Aβ oligomerization requires gangliosides that are enriched in the lipid rafts. J. Cell. Biochem. 99: 878–889, 2006.

Key words: amyloid β; lipid rafts; ganglioside

Alzheimer's disease (AD) is a neurodegenerative disease characterized by progressive impairments in both memory and cognition. A key feature of AD is the presence of amyloid plaques within the brain regions involved in learning and memory, as well as emotional behaviors. These regions include the entorhinal cortex, hippocampus, basal forebrain, and amygdale [Mattson, 2004; Tanzi and Bertram, 2005]. Amyloid plaques consist of extracellular aggregates of amyloid β peptide (Aβ), a 40–42-amino-acid fragment of the amyloid precursor protein (APP). Aβ is generated from the APP via the activity of two proteases, β- and γ-secretases [De Strooper et al., 1998; Vassar et al., 1999]. Transgenic mice expressing high levels of human mutant APP (the Swedish mutation) have been shown to develop abundant extracellular Aβ plaques, and exhibit marked synaptic loss. These results indicate that the generation of Aβ is a crucial step in the development of AD. Indeed, Aβ-mediated synaptic and neuronal dysfunction in mice has been shown to be alleviated substantially in cases in which Aβ has been neutralized with anti-Aβ antibodies [Monsonego and Weiner, 2003].

An increasing body of evidence supports the notion that cholesterol increases the incidence of AD [Kuo et al., 1998; Kivipelto et al., 2001]. Among the genes that result in familial AD, the ApoE ε4 allele is known to play some role in the elevation of cholesterol levels in the blood plasma, and hypercholesterolemia is considered to be a high risk factor for AD [Eto et al., 1988; Evans et al., 2004]. Thus, the patients who have taken the statin, a cholesterol lowering drug,

Abbreviations used: APP, amyloid precursor protein; Aβ, amyloid β; AD, Alzheimer's disease; MβCD, methyl-β-cyclodextrin

This article contains supplementary material, which may be viewed at the Journal of Cellular Biochemistry website at http://www.interscience.wiley.com/jpages/0730-2312/suppmat/index.html

Grant sponsor: Korea Research Foundation; Grant number: C00074.

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Received 10 January 2006; Accepted 31 March 2006

DOI 10.1002/jcb.20978

tend to reduce incidence of AD. This suggests a profound correlation between cholesterol and AD [Jick et al., 2000].

Lipid rafts, which are composed of glycosphingolipids and cholesterol in the plasma membrane, concentrate a host of receptors, as well as their downstream signaling molecules, thereby organizing different signal transduction sets into the specific site [Simons and Toomre, 2000; Galbiati et al., 2001; Anderson and Jacobson, 2002; Munro, 2003]. These lipid rafts are known to play roles in pathogenic invasion, cholesterol homeostasis, angiogenesis, cancer development, and neurodegenerative diseases, in addition to signal transduction [Carver and Schnitzer, 2003; Dykstra et al., 2003; Manes et al., 2003]. Since both APP and APP-cleaving enzymes, including β-secretase and γ-secretase, have been isolated from lipid raft fractions [Lee et al., 1998; Ehehalt et al., 2003; Vetrivel et al., 2004], it has been suggested that lipid rafts may be crucial to the formation of Aβ from APP. Consistent with this hypothesis, the rate of conversion from APP to Aβ is reduced significantly when the lipid rafts are disrupted by treatment with methyl-β-cyclodextrin or statin, both of which serve to reduce cholesterol levels [Simons et al., 1998; Fassbender et al., 2001; Ehehalt et al., 2003].

The Aβ generated from APP aggregated further to oligomeric and fibril forms [Podlisny et al., 1995; Yu et al., 2005]. The Aβ oligomer induces brain cell death at physiological concentrations, whereas the Aβ monomeric and fibril forms do not [Lambert et al., 1998; Klein et al., 2001; Walsh et al., 2002; Kim et al., 2003]. The Aβ oligomer also induces synaptic failure, resulting in the inhibition of long-term potentiation [Walsh et al., 2002; Cleary et al., 2005]. Thus, the Aβ oligomer might constitute a major culprit in AD. Because Aβ binds strongly with a various sialic acid-containing gangliosides and is oligomerized by ganglioside-containing liposomes [McLaurin et al., 1998; Ariga et al., 2001; Kakio et al., 2002], it has been hypothesized that the gangliosides in brain cells might be a major driving force toward the induction of Aβ oligomerization. Since gangliosides are enriched predominantly in the detergent-resistant lipid rafts [Kim et al., 2004a], it is tempting to speculate that lipid rafts might be critical sites for the accommodation of Aβ oligomerization. In this study, we have demonstrated that the Aβ oligomer appeared in the detergent-resistant lipid rafts originating from brain tissue, but not from the liver. Here, we demonstrate that gangliosides in the lipid rafts are the major propulsive force in the formation of Aβ oligomers.

MATERIALS AND METHODS

Material

Amyloid beta 1–42 ($A\beta_{42}$), methyl-β-cyclodextrin (MβCD), proteinase K, and the cholera toxin B subunit conjugated with horseradish peroxidase (CTB-HRP) were purchased from Sigma-Aldrich (Sigma, Buchs, Switzerland). The anti-Flotilin-1 antibody was obtained from Transduction Laboratories (Lexington, KY), the anti-Fyn and anti-Clathrin heavy chain antibodies were purchased from Santa Cruz Biotechnology (SantaCruz, CA), and the anti-$A\beta_{42}$ (6E10) was acquired from SIGNET Laboratories, Inc (Dedham, MA). $A\beta_{42}$ (1 mg) was treated for 1 h with 100% hexafluoro-2-propanol at room temperature, and was then aliquotted into a microcentrifuge tube. The hexafluor-2-propanol was evaporated with a Speed-Vac (Labconco Corporation, Kansas City, MO), after which the tubes were stored at $-80^{\circ}C$. An aliquot of $A\beta_{42}$ was then dissolved in anhydrous dimethyl sulfoxide (DMSO) with a final concentration to 5 mM. This $A\beta_{42}$ solution was then immediately utilized for the further experiments.

Cells and Cell Culture

CHO, CHO-K1, SK-N-MC, C_2C_{12}, and HeLa cells were purchased from the American Type Culture Collection (ATCC, Manassas, VA). CHO and CHOK1 cells were grown in Ham's Nutrient Mixture F12 supplemented with 1% penicillin/streptomycin, and 10% fetal bovine serum. The murine myoblast cell line, C_2C_{12}, the human neuroepithelioma cell line, SKNMC, and the human epithelial cell line, HeLa, were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 1% penicillin/streptomycin and 10% fetal bovine serum.

Lipid Raft Isolation

The lipid rafts were isolated as was previously described, using Triton X-100 [Kim et al., 2004b]. Rat brain tissue (300 mg) or four 150-mm dishes of cells at 90% confluence were mixed with 1 ml of lysis buffer (25 mM HEPES, pH 6.5, 150 mM NaCl, 2% TX-100, 1 mM EDTA, and

1 mM PMSF) containing a protease inhibitor cocktail (Roche Molecular Biochemicals, Indianapolis, IN), homogenized 20 times with a tight Dounce homogenizer (Kontes, Vineland, NJ), and then incubated for 20 min at 4°C. The lysate was mixed with 1 ml of 80% sucrose, transferred to an SW41Ti centrifuge tube, then overlaid with 6.5 ml of 30% sucrose solution, 3.5 ml of 5% sucrose solution containing 25 mM HEPES and 150 mM NaCl at a pH of 6.5. The discontinuous sucrose gradients were ultracentrifuged for 18 h at 4°C with an SW41Ti rotor (Beckman Instruments, Palo Alto, CA) at 200,000*g*. The gradient was then fractionated into 12 fractions, from the bottom to the top. These fractions were then used for the various kinds of experiences. Alternatively, the floating lipid raft fractions were collected carefully through peristaltic pump, diluted with 10 ml HEPES buffer (25 mM HEPES, pH 6.5, 150 mM NaCl, 1 mM EDTA, 1 mM PMSF) and centrifuged at 50,000*g* for 30 min at 4°C, allowing us to obtain a pellet consisting of detergent-resistant lipid rafts, which was then utilized in further analyses.

Treatment of Lipid Rafts With Proteinase K and Methyl-β-Cyclodextrin (MβCD)

The detergent-resistant lipid rafts (0.5 mg protein/ml) were treated with proteinase K (100 μg/ml) or 100 mM MβCD for 1 h at 37°C. In order to remove the residual proteinase K or MβCD from the lipid rafts after incubation, the protease K- or MβCD-treated lipid rafts were placed in an ultracentrifuge tube, and then overlaid with 6.5 ml 30% sucrose and 3.5 ml 5% sucrose in 25 mM HEPES, pH 6.5. The gradient was centrifuged for 1 h at 200,000*g*, at 4°C, with an SW41Ti rotor. After ultracentrifugation, the buoyant lipid rafts were collected, and incubated with 5 μM of $A\beta_{42}$ for 30 min at 4°C. The MβCD treatment was repeated three times in order to remove a greater amount of cholesterol from the lipid rafts. The concentration of cholesterol in the lipid rafts was determined with an Amplex® Red cholesterol assay kit (Molecular Probe, Carlsbad, CA).

Electrophoresis, Silver Staining, and Immunoblots

The proteins were separated on 15% polyacrylamide gel, and then visualized using a silver staining kit (Amersham Biosciences). For the immunoblotting, the proteins were transferred onto a polyvinylidene difluoride (PVDF) membrane (Millipore, Bedford, MA), using SEMI-DRY transfer cells (Bio-Rad) with transfer buffers (25 mM Tris, 192 mM glycine, 20% methanol, 0.5% SDS, pH 8.3). The membranes were then blocked in 1× TBS, 5% nonfat dry milk, and 0.5% Tween 20 for 1 h at room temperature, then allowed to react with a sequence of primary and secondary antibodies. The antigen signals were visualized using ECL reagents (Santa Cruz Biotech).

RESULTS

Aβ Is Rapidly Oligomerized in the Presence of Rat Brain Lipid Rafts

Because lipid rafts may be crucial to the formation of Aβ from APP, and gangliosides are found predominantly in the detergent-resistant lipid rafts, it is tempting to speculate that the lipid rafts might be critical sites for the induction of Aβ oligomerization. In order to address this issue, the detergent-resistant lipid rafts were isolated from the brain tissues of rats, on the basis of detergent insolubility and low density. The rat brain tissue was extracted with 1% TX-100, and then subjected to sucrose gradient ultracentrifugation, thus resulting in the formation of a distinct opaque band representing the low-density and detergent-resistant lipid rafts. The sucrose gradient was then fractionated from bottom to top, and analyzed via immunoblotting with anti-Flotillin-1, Fyn, and Clathrin heavy chain antibodies. The ganglioside-GM1 was monitored with HRP-conjugated cholera toxin B (CTB-HRP). Figure 1A demonstrates that only the raft fractions contained Flotillin-1, Fyn, and GM1, all of which are raft markers. In contrast to the raft markers, the clathrin heavy chain was found principally in the bottom fractions. Using these data, we confirmed that the detergent-resistant lipid rafts were nicely isolated from the brain tissues.

In order to verify that the detergent-resistant lipid rafts induce the formation of the $A\beta_{42}$ oligomer, we incubated the $A\beta_{42}$ with different fractions prepared after the sucrose gradient ultracentrifugation. After the incubation of each fraction for 30 min with 5 μM $A\beta_{42}$ at 4°C, each fraction was analyzed via immunoblotting with anti-Aβ antibody. Because the hydrophobic interactions within the Aβ oligomers and fibrils are too strong to be broken by SDS, SDS-resistant Aβ oligomers, and fibrils could be detected by immunoblotting with anti-Aβ anti-

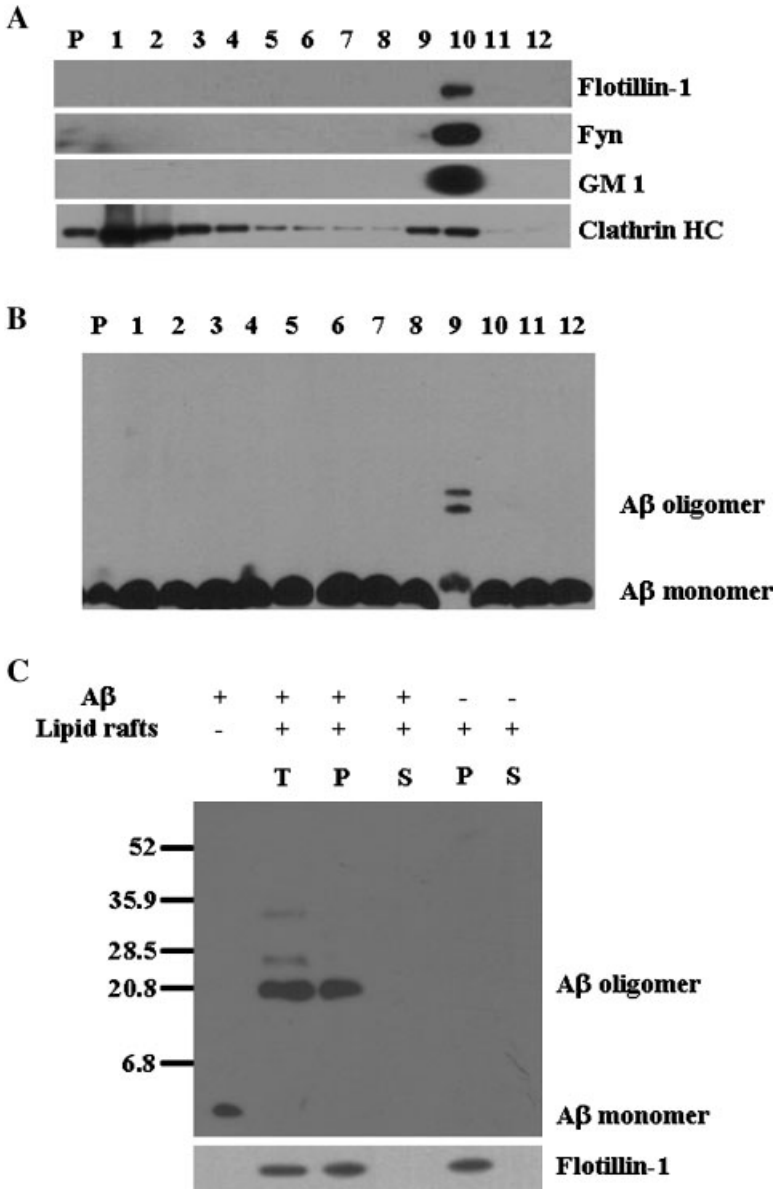

Fig. 1. Aβ is oligomerized in the presence of the rat brain lipid rafts. **A**: Detergent-resistant lipid rafts from the rat brain tissues were isolated based on their detergent-insolubility and low density. Each fraction from the sucrose gradient was immunoblotted with anti-Flotillin-1, Fyn, and Clathrin heavy chain antibodies, as well as the cholera toxin B subunit conjugated with horseradish peroxidase (CTB-HRP). P indicates the pellet after ultracentrifugation. **B**: Lipid rafts from rat brain tissue were isolated in the presence of Triton X-100. The sucrose gradient was fractionated from top to bottom. Each of the fractions (100 μl) was incubated with 5 μM of $A\beta_{42}$, and then analyzed by immunoblotting with anti-Aβ antibody. **C**: $A\beta_{42}$ (5 μM) was incubated with 20 μl of rat brain lipid rafts (0.5 mg protein/ml) at 4°C. Thirty minutes after incubation, the total extract was microcentrifuged for an additional 20 min at 20,000*g*. Total extract (T), pellet (P), and supernatant (S) were analyzed by immunoblotting with anti-Aβ antibody.

body after SDS–PAGE [Podlisny et al., 1995]. As shown in Figure 1B, the lipid raft fraction was implicated in the conversion of the Aβ monomer to oligomers, whereas the other fractions were not. In order to determine whether the Aβ oligomers were associated with the lipid rafts, the lipid rafts were precipitated by ultracentrifugation after incubated with $A\beta_{42}$. The Aβ was analyzed from the pellet and supernatant via immunoblotting. It should be noted that isolated lipid rafts can be precipitated fairly easily after ultracentrifugation, due to their membrane structure [Bae et al., 2004]. Figure 1C shows that the Aβ oligomers (mainly tetramer) were observed in the pellets, but not in the supernatant, demonstrating that the

Aβ oligomers were associated with the lipid rafts.

We then attempted to determine whether Aβ oligomerization was dependent on brain-specific lipid rafts or not. Detergent-resistant lipid rafts were isolated from both the brain and liver tissues of rats in the presence of 1% Triton X-100. Each of the lipid raft sample was then incubated with $A\beta_{42}$ at the indicated concentrations for 30 min at 4°C, and then the levels of Aβ monomers and oligomers were measured by immunoblotting with anti-Aβ antibody. As is shown in Figure 2, the brain lipid rafts induced Aβ oligomerization to a significant degree, even at low protein concentrations (0.05 mg/ml protein), whereas the liver lipid rafts oligomerized Aβ only to a minimal degree. These results indicate that brain lipid rafts have stronger activity to oligomerize Aβ than liver lipid rafts have.

Stability of Raft-Induced Aβ Oligomers

Because the Aβ oligomers have been reported to aggregate further into Aβ fibrils, we attempted to determine the effects of temperature and time on the stability of the raft-induced Aβ oligomers. The lipid raft samples were incubated with $A\beta_{42}$ for 30 min at 4°C, 24°C, and 37°C, and analyzed by immunoblotting with anti-Aβ antibody. As is shown in Figure 3A, the raft-induced Aβ oligomers did not aggregate further into the fibrils at any temperatures. This indicates that the Aβ oligomers are remarkably stable, even at physiological temperature. We then determined the time-dependency characteristics in the stability of the raft-induced Aβ oligomers. After the incubation of the lipid rafts with $A\beta_{42}$ for various times (0, 0.5, 1, 2, 4, and 24 h), Aβ oligomers were analyzed by immunoblotting. Figure 3B shows that the Aβ oligomers aggregated into few Aβ fibrils, even at longer incubation times. Aβ fibrils were discovered only at minimal levels, even after 4 days of incubation (data not shown), indicating that the raft-induced Aβ oligomers are remarkably structurally stable. In addition, it should be noted that the Aβ oligomers appeared immediately after the incubation of the lipid rafts with Aβ (Fig. 3B).

Next, we found that Aβ fibrils were rarely present, even after the incubation of high concentrations of Aβ (5 μM) with the lipid raft samples (Fig. 3C), demonstrating that the raft-induced Aβ oligomers were quite stable. Moreover, as shown in Figure 3C, raft-dependent Aβ oligomers were also detected after incubation, even at low Aβ concentrations (as low as 100 nM).

Proteins and Cholesterol in the Lipid Rafts Are not Required for Aβ Oligomerization

In order to characterize the Aβ oligomer-inducing factors within the lipid rafts, we modified the lipid rafts with various treatments.

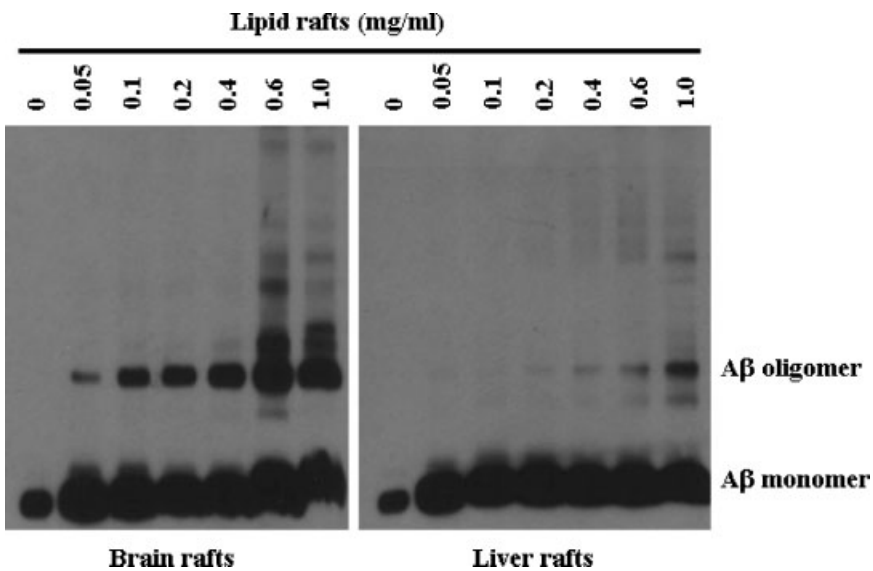

Fig. 2. Brain lipid rafts induce Aβ oligomerization more strongly than do liver lipid rafts. Lipid rafts were isolated from rat brain and liver in the presence of Triton X-100. Lipid rafts (20 μl) at different concentrations (0, 0.05, 0.1, 0.2, 0.4, 0.6, and 1.0 mg protein/ml) were incubated with 5 μM of $A\beta_{42}$ at 4°C. Thirty minutes after incubation, the total extracts were analyzed by immunoblotting with anti-Aβ antibody.

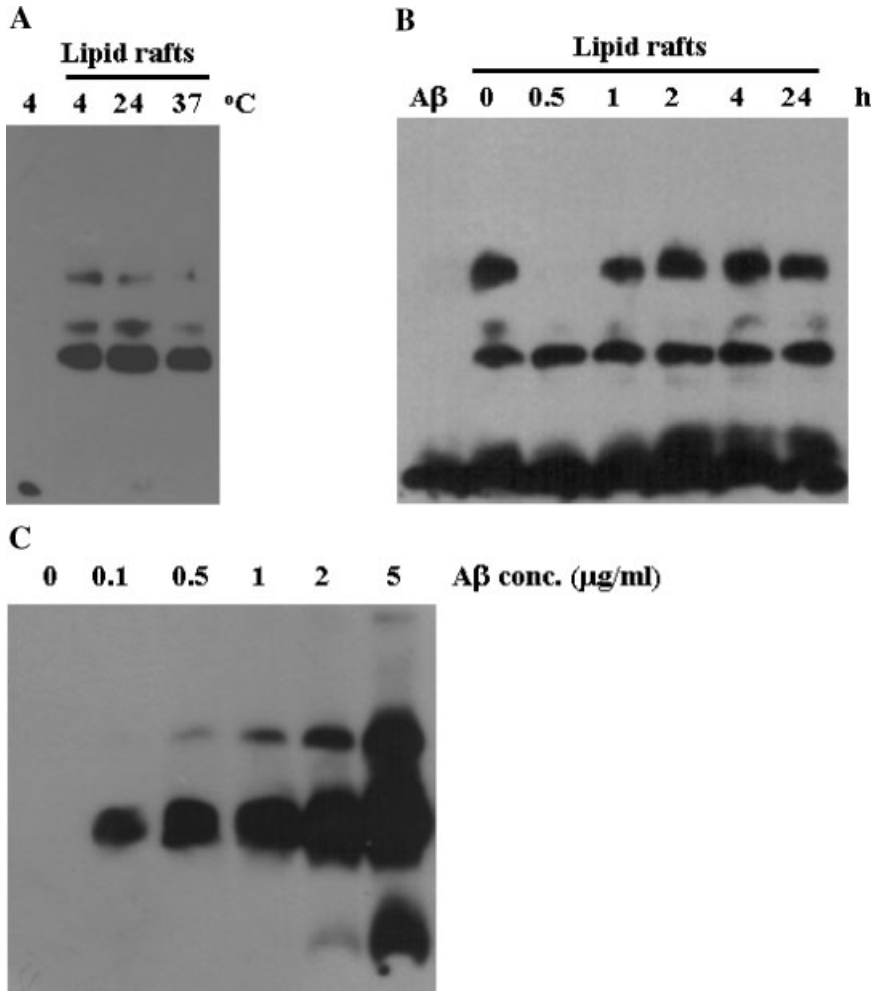

Fig. 3. Brain lipid raft-induced Aβ oligomers do not aggregate into Aβ fibers even after long incubation periods at physiological temperature. **A**: Rat brain lipid rafts (20 μl) (0.5 mg protein/ml) were incubated with 5 μM of $Aβ_{42}$ for 30 min at 4, 24, and 37°C, respectively. **B**: Rat brain lipid rafts (20 μl) (0.5 mg protein/ml) were incubated with 5 μM of $Aβ_{42}$ at 4°C for 0, 0.5, 1, 2, 4, and 24 h. **C**: Rat brain lipid rafts (20 μl) (0.5 mg protein/ml) were incubated with different concentrations of Aβ (0, 0.1, 0.5, 1, 2, and 5 μM) for 30 min at 4°C. Total mixtures were analyzed by immunoblotting with anti-Aβ antibody.

Lipid rafts were boiled for 20 min with or without 1% SDS for denaturing proteins, or sonicated 6 times for 10 s with 30 s of intervals for disrupting lipid raft structure. Modified lipid rafts were incubated with $Aβ_{42}$ for 30 min at 4°C, and then the level of Aβ oligomers was determined by immunoblotting. As shown in Figure 4A, Aβ oligomerization was not affected by heat, SDS, or sonication, indicating that the proteins themselves are not the factors that induce the oligomerization of Aβ. Next, the lipid rafts were treated for 1 h with protease K, in order to digest all of the lipid raft proteins. The left panel in Figure 4B shows that the lipid raft proteins had been digested completely by the protease K in the silver-stained gel. Interestingly, the degree to which Aβ was oligomerized was not affected when $Aβ_{42}$ was incubated with lipid rafts treated with protease K (Fig. 4B, right panel), thus confirming that lipid raft proteins are not crucial to the oligomerization of Aβ.

In order to characterize the effects of cholesterol on raft-mediated Aβ oligomerization, we removed the cholesterol from the lipid rafts by treating the rafts with methyl-β-cyclodextrin (MβCD). The lipid rafts were treated for 1 h with 100 mM MβCD at 37°C. After this MβCD treatment, the lipid rafts were re-isolated via sucrose gradient ultracentrifugation. The re-isolated lipid rafts were then re-treated with 100 mM MβCD two times. Figure 5A shows the

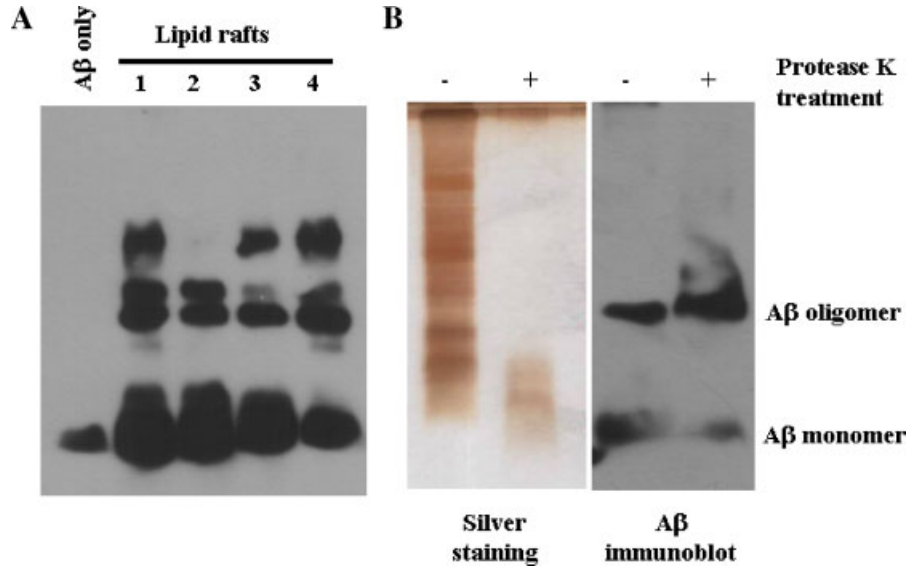

Fig. 4. Lipid raft proteins are not essential to Aβ oligomerization. **A**: Rat brain lipid rafts (20 μl) (0.5 mg protein/ml) were boiled for 30 min with or without SDS-sample buffer (**lane 2** and **lane 3**, respectively), or sonicated six times for 10 s with 30-s of intervals (**lane 4**). **Lane 1** showed the sample without any lipid rafts. These lipid rafts were incubated with 5 μM of Aβ at room temperature, and the total proteins were analyzed via immunoblotting with anti-Aβ antibody. **B**: Rat brain lipid rafts (20 μl) (0.5 mg protein/ml) were treated for 1 h with proteinase K (100 μg/ml) at 37°C. After being treated with proteinase K, the lipid rafts were further separated via discontinuous sucrose gradient ultracentrifugation. The buoyant lipid rafts were incubated with 5 μM of $A\beta_{42}$ for 30 min at 4°C, and then analyzed via immunoblotting with anti-Aβ antibody (**Right panel**). The **left panel** shows that the lipid raft proteins were degraded completely after proteinase K treatment, as evidenced by silver staining. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

cholesterol levels observed after treating the rafts with 100 mM MβCD. The cholesterol concentration in the lipid rafts decreased dramatically from 432 μg/ml to 22 μg/ml after the lipid rafts had been treated three times with MβCD. Lipid rafts containing varying cholesterol contents were then incubated with $A\beta_{42}$, and analyzed by immunoblotting with anti-Aβ antibody. Figure 5B shows that the rate of raft-mediated Aβ oligomerization was not altered by the removal of cholesterol by MβCD, thereby suggesting that cholesterol could be excluded as

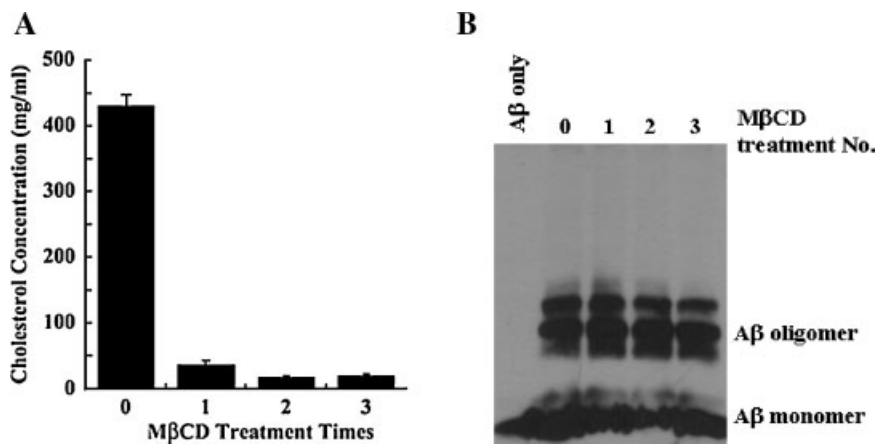

Fig. 5. Cholesterol in brain lipid rafts is not essential to Aβ oligomerization. **A**: Cholesterol concentrations in the lipid rafts after brain lipid rafts had been treated with methyl-β-cyclodextrin (MβCD). Rat brain lipid rafts (20 μl) (0.5 mg protein/ml) were treated for 1 h with 100 mM MβCD at 37°C. After the initial MβCD treatment, the lipid rafts were isolated further via discontinuous sucrose gradient ultracentrifugation. In order to remove a greater quantity of cholesterol, the buoyant lipid rafts were treated with MβCD twice more, as above. The cholesterol content from each of the lipid raft samples was measured with a cholesterol assay kit. This experiment was done three times. **B**: Brain lipid rafts with different cholesterol contents were treated with 5 μM $A\beta_{42}$ for 30 min at 4°C, then analyzed via immunoblotting with anti-Aβ antibody.

a factor in the induction of Aβ oligomerization by the lipid rafts.

Gangliosides Are Required for Lipid Raft-Mediated Aβ Oligomerization

Because brain lipid rafts contained much higher GM1 concentration than liver lipid rafts do (data not shown), and Aβ exhibits a strong interaction with GM1 [Kakio et al., 2002], it is tempting to speculate that the gangliosides in lipid rafts might be required for the raft-mediated oligomerization of Aβ. In order to address the issue, we selected different cells, with differing ganglioside contents. For example, the premyocyte C_2C_{12} cells harbor abundant gangliosides, whereas the neuroblastoma (SK-N-MC) and HeLa cells contain a relatively low level of gangliosides [Gillard et al., 1998]. The lipid rafts from the C_2C_{12}, HeLa, and SK-N-MC cells were incubated with $A\beta_{42}$, and then analyzed by immunoblotting with anti-Aβ antibody. Figure 6A shows that the lipid rafts from the C_2C_{12} cells potently induced Aβ oligomerization, whereas the lipid rafts from the HeLa and SK-N-MC cells did not. This clearly suggests that the gangliosides in the lipid rafts might be the salient factor with regard to the raft-mediated induction of Aβ oligomerization. Silver staining data in Figure 6B shows that the same amounts of raft proteins were loaded in each of the samples.

In order to firmly verify the involvement of gangliosides in the raft-mediated oligomerization of Aβ, the lipid rafts isolated from ganglioside-poor CHO-K1 cells were also incubated with Aβ. Because CHO-K1 cells are genetically deficient with regard to the synthesis of complex gangliosides, CHO-K1 cells are free of most gangliosides, including GM1, GM2, GD3, GD1a, and GT3, although they do harbor some GM3 [Crespo et al., 2002; Rusnati et al., 2002], Lipid rafts were isolated from the CHO and CHO-K1 cells, and analyzed by immunoblotting with anti-Flotillin-1 antibody. As shown in Figure 6C, both cells had the detergent-resistant lipid rafts with the enrichment of Flotillin-1. Both lipid rafts were incubated with Aβ (0.1 mg/ml raft proteins), and evaluated via immunoblotting. Figure 6D demonstrates that Aβ was oligomerized normally by the lipid rafts isolated from the normal CHO cells. Interestingly, however, the level of Aβ oligomers were reduced in cases in which the lipid rafts used in the incubations had been isolated from ganglioside-poor CHO-K1 cells. This suggests that gangliosides might be one of the primary factors in the induction of raft-mediated Aβ oligomerization. However, it should also be noted that the oligomerization of Aβ was not completely abolished by the lipid rafts from the CHO-K1 cells, and this may be due to the fact that the CHO-K1 cells contain GM3, which can also bind to Aβ. With all of the data shown in Figure 6, we were able to conclude that the gangliosides in the lipid rafts were, indeed, the critical factor in the raft-mediated induction of Aβ oligomerization.

DISCUSSION

The Aβ peptide is known to self-aggregate into Aβ fibrils in AD patients. Because Aβ fibrils induce neuronal cell death at μM level concentration, Aβ fibrils have long been considered to be the primary culprit in the development of AD [Hardy and Higgins, 1992]. However, controversy has raged with regard to the Aβ fibril-induced development of AD. In cases of AD, the observed sites of neuronal loss do not precisely match with the amyloid plaques, which predominantly contain Aβ fibrils [Katzman et al., 1988]. In addition, the Aβ load itself does not appear to result in neuronal damage, nor does it seem to induce synaptic terminal loss [Klein et al., 2001]. Therefore, soluble Aβ oligomers, rather than the Aβ fibrils, might actually be the principal causative factor of AD, because they do induce neuronal cell death in physiological concentration, and even at pM concentrations [Lambert et al., 1998; Zhang et al., 2002]. Aβ oligomers are often isolated in AD patients in whom amyloid plaques cannot be identified. Moreover, Aβ oligomers result in the loss of long-term potentiation, and also induce disruptions in cognitive functioning [Cleary et al., 2005; Kayed et al., 2003; Walsh et al., 2002].

Our findings suggest that the monomeric form of Aβ self-aggregates into Aβ oligomers in the presence of detergent-resistant lipid rafts isolating from the brain tissues of rats. The monomeric form of Aβ is, in fact, converted very rapidly into the tetrameric form of Aβ by the brain lipid rafts. Aβ dimers and trimers have been observed in many in vitro Aβ oligomerization experiments, but in our study, the lipid rafts isolated from the rat brains appeared to induce the formation of Aβ tetramers. Because the tetrameric form of Aβ is encountered

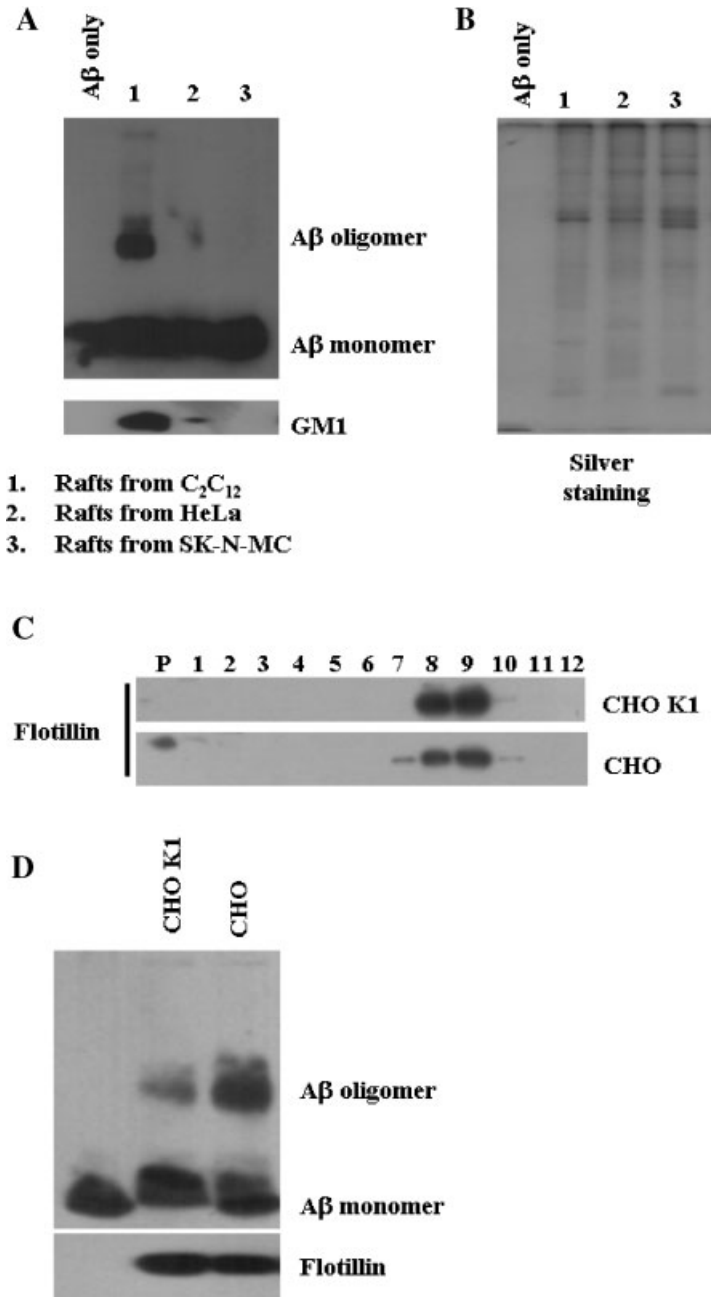

Fig. 6. Ganglioside levels in the lipid rafts are important determinants of Aβ oligomerization. **A**: Lipid rafts were isolated from C_2C_{12}, HeLa, and SKNMC cells. It should be noted that C_2C_{12} is a ganglioside-rich cell, whereas the HeLa and SKNMC cells are ganglioside-poor cells. Lipid rafts (20 μl) (0.5 mg protein/ml) were incubated for 30 min with 5 μM $A\beta_{42}$ at 4°C, and analyzed by immunoblotting with anti-Aβ antibody. In addition, each raft was blotted with CTB-HRP (**bottom panel**) to determine the concentration of GM1. **B**: In order to confirm that an equal amount of lipid raft proteins were loaded, the lipid raft proteins were electrophoresed and silver-stained. **C**: The lipid rafts were isolated from CHO-K1 (ganglioside-poor cells) and CHO cells. Each fraction from the sucrose gradient was analyzed via immunoblotting with anti-flotillin. **D**: Lipid rafts (20 μl) (0.5 mg protein/ml) from the CHO-K1 and CHO cells were incubated for 30 min with 5 μM $A\beta_{42}$ at 4°C, then analyzed via immunoblotting with anti-Aβ antibody.

principally in the brains of AD patients, brain lipid raft-induced Aβ tetramers might also be a major culprit in the development of AD [Lambert et al., 2001]. It was reported in a study of the aggregation of Aβ in a mixture of Aβ and lipid raft-like liposomes, via circular dichroism and atomic force microscopy, that Aβ was eventually converted to fibrils, after longer

periods of incubation [McLaurin et al., 1998; Zhu et al., 2004]. However, in our study, lipid raft-induced Aβ tetramers aggregate into few Aβ fibrils, even after 4 days of incubation.

Because lipid rafts harbor both cholesterol and gangliosides, lipid raft-dependent Aβ oligomerization might be resultant from the activities of both cholesterol [Walsh et al., 2002] and gangliosides [Yanagisawa and Matsuzaki, 2002]. Since brain lipid rafts had stronger activity to oligomerize Aβ (Fig. 2), and contained higher ganglioside amount than liver lipid rafts did (data not shown), gangliosides could be a strong inducer to oligomerize Aβ. Indeed, our data unambiguously demonstrate that gangliosides, but not cholesterol, might be a major component in the induction of Aβ oligomerization, as a lesser amount of Aβ oligomers was formed in the lipid rafts isolated from the ganglioside-poor cells (Fig. 6), and cholesterol removal from the lipid rafts had no significant effects on the oligomerization of the Aβ (Fig. 5). This finding was quite consistent with other recently reported data, which pointed to a significant increase in gangliosides and decrease in cholesterol levels in the brains of AD patients [Pitto et al., 2005]. Thus, Aβ can be oligomerized by the increased levels of gangliosides often detected in AD patients, consequently inducing an Aβ oligomer-mediated neuronal loss.

The ganglioside-poor cells used in our study, the CHO-K1 cells, still evidenced some ability to oligomerize Aβ, suggesting that there might have a ganglioside-independent mechanism for Aβ oligomerization. Therefore, in order to determine precisely the effects of gangliosides on Aβ oligomerization, we treated the cells with fumonisin B1, a ganglioside synthesis inhibitor, to remove the gangliosides from the cells, which were then allowed to react with Aβ. In this experiment, however, the rates at which Aβ was oligomerized were not dramatically reduced (data not shown), thereby suggesting that a ganglioside-independent Aβ oligomerization mechanism may, in fact, exist. In addition to the gangliosides, other protein components have been implicated in Aβ oligomerization. For example, ApoJ, clusterin, and hemoglobin all appear to induce either Aβ oligomerization or fibrillization [DeMattos et al., 2002; Lambert et al., 1998; Wu et al., 2004]. However, because Aβ oligomers were clearly formed by the protease-treated lipid rafts, lipid raft proteins cannot be the actual components that induce Aβ oligomerization. Instead, specific lipid components or carbohydrates in the lipid rafts might function as potent activators of the conversion of monomeric Aβ to Aβ oligomer. Thus, it is necessary to determine non-protein, and non-ganglioside components that induce Aβ oligomerization by further investigation.

ACKNOWLEDGMENTS

This work was supported by a grant from the Korea Research Foundation (C00074) to Y.-G. Ko.

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