Accelerated Aβ aggregation in the presence of GM1-ganglioside-accumulated synaptosomes of aged apoE4-knock-in mouse brain

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Abstract Aging and apolipoprotein E4 (apoE4) expression are strong risk factors for the development of Alzheimer's disease (AD); however, their pathological roles remain to be clarified. In the process of AD development, the conversion of the nontoxic amyloid -protein (A) monomer to its toxic aggregates is a fundamental process. We previously hypothesized that A aggregation is accelerated through the generation of GM1 ganglioside (GM1)-bound A which acts as a seed for A fibril formation. Here we report that GM1 level in detergent-resistant membrane microdomains (DRMs) of synaptosomes increased with age and that this increase was significantly pronounced in the apoE4- than the apoE3-knock-in mouse brain. Furthermore, we show that A aggregation is markedly accelerated in the presence of the synaptosomes of the aged apoE4-knock-in mouse brain. These observations suggest that aging and apoE4 expression cooperatively accelerate A aggregation in the brain through an increase in the level of GM1 in neuronal membranes.

Keywords: Amyloid β-protein; Apolipoprotein E; Ganglioside; Alzheimer's disease; Lipid raft

1. Introduction

Apolipoprotein E (apoE) allele epsilon4 is the strongest genetic risk factor for the development of Alzheimer's disease (AD) [1–4]. Previous studies suggested that apoE isoforms modulate aggregation [5,6], clearance [7,8] and metabolism [9] of the amyloid β-protein (Aβ), a proteinaceous component of senile plaques, through apoE and Aβ interaction. Alternatively, it was also suggested that apoE isoforms influence neurite extension [10,11], neuronal integrity [12] and antioxidant ability [13].

On the basis of results obtained from studies using recombinant human apoE isoforms and human apoE-knock-in mice [14], we suggested that apoE modulates lipid efflux from neurons [15] and cholesterol distribution in the synaptic plasma membranes [16] in an isoform-dependent manner. Despite these findings, it remains to be elucidated whether putative apoE-isoform-dependent alterations in the metabolism and/or distribution of lipids in neuronal membranes are associated with AD development, particularly with the conversion of nontoxic Aβ monomer to its toxic aggregates.

Regarding the mechanism underlying Aβ aggregation in the brain, we previously hypothesized that Aβ adopts an altered conformation through its binding to GM1 ganglioside (GM1) and that the GM1-bound Aβ acts as a seed for Aβ fibrillogenesis [17]. In the present study, we investigated whether aging and expression of different apoE isoforms modulate the level of GM1 in neuronal membranes, particularly in detergent-resistant membrane microdomains (DRMs), and also whether such an alteration in the level of GM1, if any, influences Aβ aggregation in vitro.

2. Materials and methods

2.1. Animals

Three different age groups of male homozygous epsilon3 (3/3) and epsilon4 (4/4) knock-in mice, in which a portion of the *Apoe* gene was replaced with human apoE3 or apoE4 cDNA [14], were used in this study.

2.2. Materials

Streptavidin-horseradish peroxidase conjugate (avidin-HRP) was purchased from Amersham Bioscience (Piscataway, NJ). Cholera toxin B subunit-horseradish peroxidase conjugated (CTX-HRP) and methyl-β-cyclodextrin (MbCD) were obtained from Sigma–Aldrich (St. Louis, MO). Anti-flotillin monoclonal antibody (anti-flotillin-1), anti-prion protein (PrP) antibody [PrP(C-20)] and anti-Aβ monoclonal antibody (4G8) were purchased from Transduction Laboratories (Lexington, KY), Santa Cruz Biotechnology (Santa Cruz, CA) and Signet Laboratories (Dedham, MA), respectively. Biotinylated derivative of perfringolysin O (BCθ) was prepared as previously described [18]. Bicinchoninic acid (BCA) protein assay kit was purchased from Pierce (Rockford, IL).

2.3. Preparation of DRMs from synaptosomes

Synaptosomes were prepared from whole brain as previously reported [19]. DRMs were prepared from the synaptosomes according to

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a previously established method [20]. Briefly, synaptosomes (0.1 mg protein/ml) were homogenized in MES-buffer saline (MBS) containing 1% Triton X-100. The sucrose concentration of the extract was adjusted to 40% by the addition of 80% sucrose in MBS, and then they were overlaid with a 5%/35% discontinuous sucrose gradient in MBS without Triton X-100, followed by centrifugation at 39 000 rpm for 20 h in an SW41-Ti rotor (Beckman, Palo Alto, CA). After centrifugation, 1-ml fractions were harvested from the top to the bottom of the gradient.

2.4. SDS–PAGE and gel blotting

The proteins of each fraction were separated by SDS–polyacrylamide gel electrophoresis (SDS–PAGE), transferred onto NitroBind membrane (GE Osmonics, Minnetonka, MN) and visualized using ECL system (Amersham Bioscience). For detection of cholesterol-rich domain, synaptosomes were incubated with BCθ [18] (5 μg/ml) in PBS containing 1 mg/ml BSA at 25 °C for 20 min, and washed twice with the same volume of PBS to remove unbound BCθ. The precipitates were subjected to fractionation for DRMs. The same volume of each fraction was analyzed by SDS–PAGE. BCθ in the gels was transferred onto NitroBind membrane and was detected with avidin-HRP. Quantitative scanning of the blots were performed using NIH Image version 1.59.

2.5. MbCD treatment

The synaptosomes were washed with Tris-buffered saline (TBS1; 20 mM Tris–HCl, 150 mM NaCl, pH 7.4) at 4 °C and then resuspended in TBS1 with or without 10 mM MbCD. After incubation at 37 °C for 30 min, the mixture was centrifuged at 17 000 × *g* for 15 min. The resultant precipitates were subjected to fractionation for DRMs.

2.6. Aβ incubation in the presence of synaptosomes

Seed-free solutions of Aβ (Aβ1–40) (Peptide Inst., Osaka, Japan) were prepared essentially according to a previous report [21]. Aβ solutions at 50 μM in Tris-buffered saline (TBS2: 150 mM NaCl and 10 mM Tris–HCl, pH 7.4) were incubated at 37 °C with or without synaptosomes. Thioflavin T (ThT) fluorescence intensities in the mixture incubated for 24 h were determined as previously described [21].

3. Results

3.1. GM1 levels in DRMs of synaptosomes

We isolated DRMs from synaptosomes prepared from three different age groups (8 weeks, 1 and 2 years) of apoE3- and apoE4-knock-in mice. To determine the levels of GM1 in DRMs, we incubated the blots of these fractions with CTX-HRP. The levels of CTX, a highly specific and sensitive for GM1 ganglioside [22], bound to DRMs isolated from apoE4-knock-in mouse brain synaptosomes markedly increased with age (Fig. 1). The age-dependent increase in the level of CTX binding to DRMs was also observed on the blots of apoE3-knock-in mouse brain synaptosomes; however, it was not as marked as in that of apoE4-knock-in mice (Fig. 1). To explore the nature of age-dependent increase in CTX-binding to DRMs, we performed further experiments using apoE4-knock-in mouse brains.

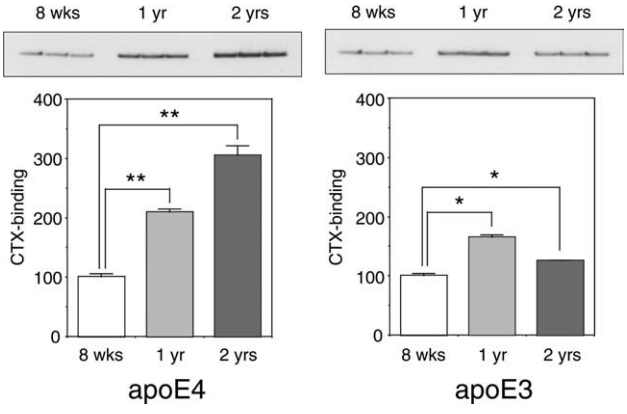

Fig. 1. CTX binding to the blots of DRM fractions isolated from brain synaptosomes from three different age groups of apoE3- and apoE4-knock-in mice. Each lane contains 0.1 μg protein of DRM fraction isolated from synaptosomes prepared from five brains combined. The blots were reacted with CTX-HRP. The intensities of the bands relative to those for 8 weeks old mice are indicated. Each column represents the average value ± 1S.D. of three values $^{**}P < 0.0001$, $^{*}P < 0.001$ (one-way ANOVA combined with Sheffe's test).

3.2. Characterization of age-dependent GM1 accumulation in DRMs

The levels of CTX bound to the DRM fraction (fraction 5) markedly increased with age whereas those bound to other

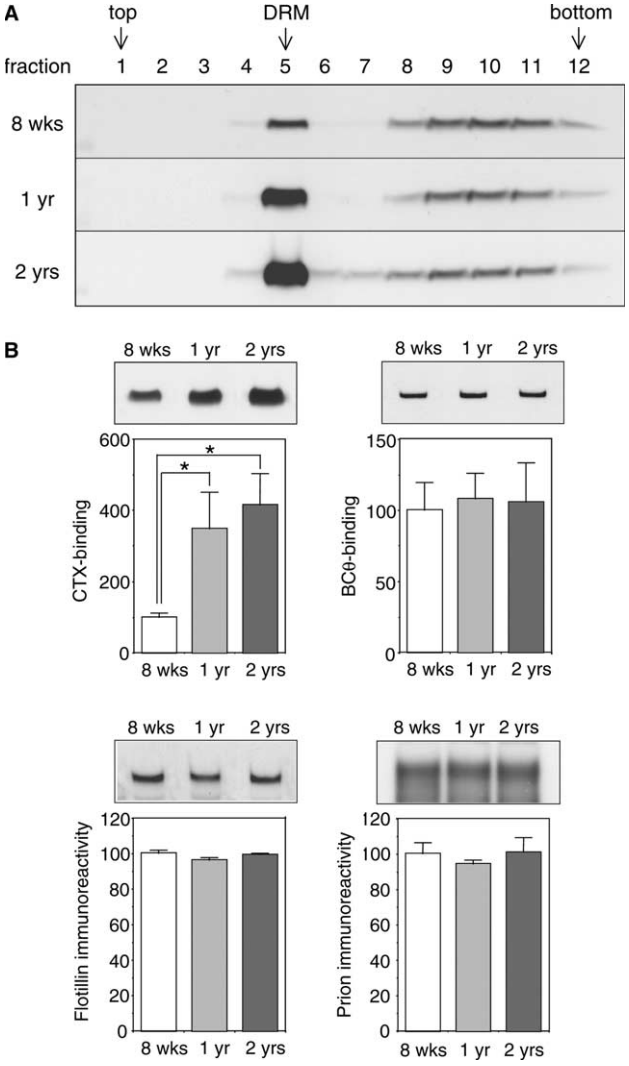

Fig. 2. Properties of the DRMs isolated from brain synaptosomes from three different age groups of apoE4-knock-in mice. (A) CTX binding to the blots containing all sucrose density gradient fractions of synaptosomes. (B) Binding of CTX, anti-flotillin-1 and PrP(C-20) to the blots of DRM fractions. To examine the levels of BCθ bound to DRMs, synaptosomes were incubated with BCθ prior to fractionation. The blots of BCθ-labeled fractions were incubated with avidin-HRP. The intensities of the bands relative to those for 8 weeks old mice are indicated. Each column represents the average value ± 1S.D. of four values. $^{*}P < 0.001$ (one-way ANOVA combined with Sheffe's test).

cellular membranes (fractions 8–12) were apparently unchanged with age (Fig. 2A).

To investigate whether the number or size of DRMs increases with age, we incubated the synaptosomes with BCθ, which specifically binds to cholesterol-rich microdomains such as lipid rafts [18], prior to the fractionation. We also performed Western blotting of DRM fractions with antibodies specific to flotillin or PrP, which are selectively localized in DRMs [23,24]. Notably, there was no apparent age-dependent increase in the levels of BCθ, anti-flotillin-1 and PrP(C-20) bound to the DRM fractions (Fig. 2B). These results suggest that the age-dependent GM1 accumulation was not due to an increase in the number or size of DRMs.

To further characterize the GM1 accumulation in DRMs, we treated the synaptosomes with MbCD, which can potently break down cholesterol-rich DRMs by removing cholesterol, prior to fractionation. Following MbCD treatment, the levels of BCθ binding markedly decreased in all the fractions (Fig. 3A). However, the levels of CTX bound to the DRM fraction were essentially unchanged following the MbCD treatment (Fig. 3A). The levels of cholesterol in all the fractions markedly decreased to below detection limits following the MbCD treatment (Fig. 3B). Interestingly, a population of phospholipids was still recovered in the DRM fraction (Fig. 3B). The total protein levels in each fraction were essentially unchanged following the MbCD treatment (Fig. 3C). Taken together, these results suggest that GM1 accumulates in a distinct subset of DRMs, which is different from MbCD-sensitive DRMs.

3.3. *Aβ aggregation in the presence of synaptosomes of apoE4 mouse brain*

Finally, we investigated whether GM1 accumulation in the DRMs of synaptosomes can potently accelerate Aβ aggregation. We incubated soluble Aβ1–40 in the presence of the synaptosomes. The fluorescence intensity of ThT, which specifically recognizes the amyloid structure, in the incubation mixtures significantly increased with age of the mice (Fig. 4A). We performed Western blotting of both the precipitates and supernatants of the incubation mixtures following ultracen-

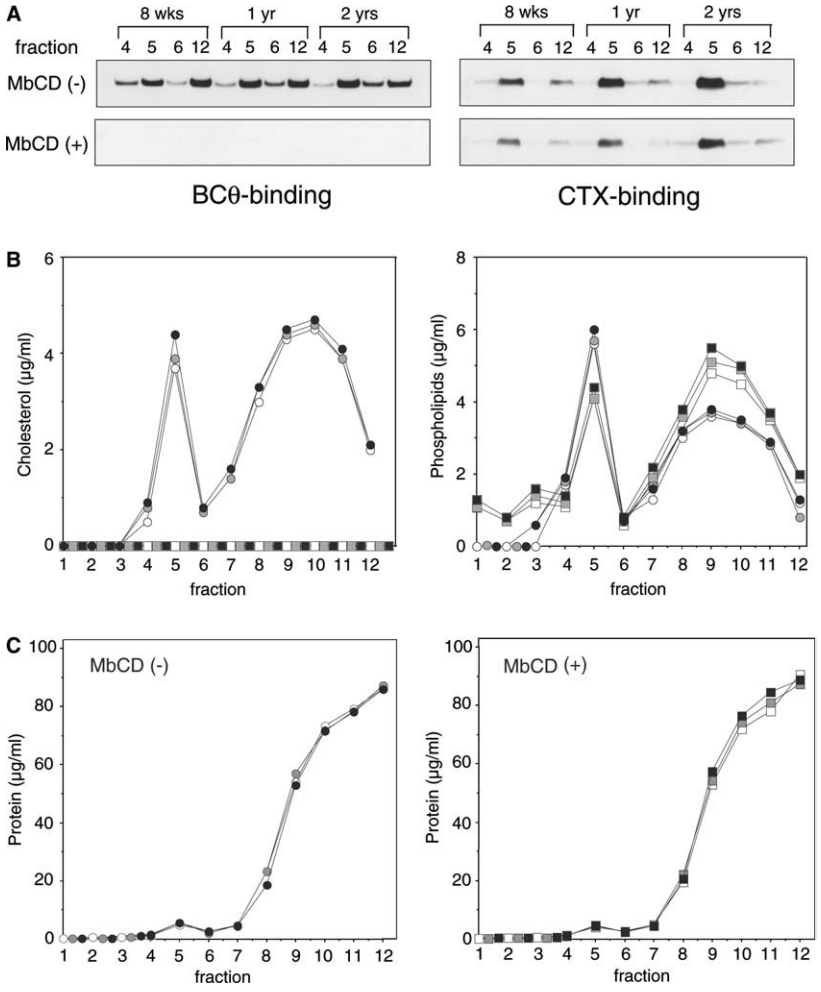

Fig. 3. MbCD treatment of synaptosomes prepared from three different age groups of apoE4-knock-in mice. (A) Left panel: The blots of BCθ-labeled sucrose density gradient fractions, probed with avidin-HRP. BCθ-labeling was performed as described in the Fig. 2 legend. Right panel: The blots of fractions, probed with CTX-HRP. (B) The levels of cholesterol and phospholipids in each fraction were determined using Determiner L (Kyowa, Tokyo, Japan) and Phospholipids C (Wako, Osaka, Japan), respectively. (C) The total protein levels in each fraction were determined using BCA protein assay kit. Open, shaded and closed symbols indicate the values of 8 weeks, 1 and 2 years old mice, respectively. Circle and square indicate the values without and with MbCD treatment of synaptosomes, respectively.

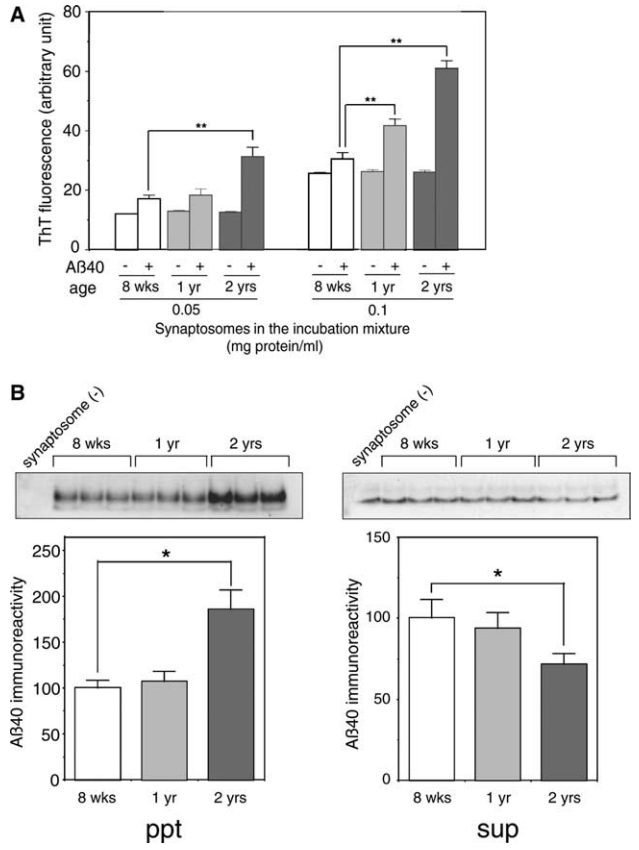

Fig. 4. Aβ aggregation in the presence of synaptosomes prepared from brains of three different age groups of apoE4-knock-in mice. Aβ solutions were incubated at 50 μM and 37 °C for 24 h in the presence or absence of the synaptosomes. (A) ThT fluorescence intensity in the incubation mixtures of synaptosomes with or without Aβ. Each column represents the average ± 1S.D. of four values. (B) Western blots of the precipitates (ppt) and supernatants (sup) of the incubation mixtures. The materials precipitated from 100 μl of incubation mixture were subjected to SDS–PAGE following solubilization in formic acid. The Aβ in the supernatant was subjected to SDS–PAGE following dilution with 200-fold volume of incubation buffer. The blots were reacted with 4G8. The intensities of the bands relative to those for 8 wks old mice are indicated. Each column represents the average ± 1S.D. of three values. $^{**}P < 0.0001$, $^{*}P < 0.05$ (one-way ANOVA combined with Sheffe's test).

trifugation at 540 000 × *g* for 15 min. A significant increase in the levels of Aβ immunoreactivities in the precipitates, and a concomitant decrease in those in the supernatants were observed with age of the mice (Fig. 4B).

4. Discussion

In the present study, we suggest that aging and apoE4 expression cooperatively accelerate Aβ aggregation in the brain through an increase in the level of GM1 in particular microdomains of neuronal membranes. Fagan et al. have recently reported that Aβ is accumulated in detergent-insoluble glycolipid-enriched membrane domains (DIGs), corresponding to DRMs, in the brain of amyloid precursor protein (APP) transgenic mice expressing human apoE isoforms [25]. Taken together with previous reports of preferential Aβ accumulation in DIGs in vivo [26–29], it seems likely that Aβ accumulation in these microdomains is an early event of the process of AD development. Although further studies are required, the results of previous [17,30,31] and present studies suggest that GM1-bound Aβ, an endogenous seed for Aβ fibrillogenesis, is generated in these microdomains.

Regarding biochemical features of membrane microdomains, including lipid rafts and caveolae, evidence has been accumulating to suggest that these microdomains are heterogenous [32–34]; there is a subset of microdomains that are rich in gangliosides with low levels of cholesterol [32]. Our results suggest that GM1 accumulates in unique microdomains in an aging- and apoE-expression-dependent manner.

At present, it remains to be elucidated how GM1 accumulates in such microdomains in aging- and apoE4-expression-dependent manner. We previously found that cholesterol level in the exofacial leaflet of synaptic plasma membranes increases with age [19] and by apoE expression [16,35]. We also reported that the accumulation of cholesterol in membranes can induce the GM1 clustering [36]. Thus, one of the possible explanations is that aging and apoE4 expression stabilize GM1 in the particular membrane microdomains. An alternative explanation may be that both aging and apoE4 expression directly modulate trafficking of GM1 so that GM1 level in a subset of microdomains increases.

In conclusion, our results suggest that one of the pathological roles of aging and apoE4 expression is acceleration of Aβ aggregation through modulation of GM1 distribution in neuronal membranes.

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