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Review

Role of gangliosides in Alzheimer's disease

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Abstract

One of the fundamental questions regarding the pathogenesis of Alzheimer's disease (AD) is how the monomeric, nontoxic amyloid β-protein (Aβ) is converted to its toxic assemblies in the brain. A unique Aβ species was identified previously in an AD brain, which is characterized by its binding to the GM1 ganglioside (GM1). On the basis of the molecular characteristics of this GM1-bound Aβ (GAβ), it was hypothesized that Aβ adopts an altered conformation through its binding to GM1, and GAβ acts as a seed for Aβ fibrillogenesis in an AD brain. To date, various *in vitro* and *in vivo* studies of GAβ have been performed, and their results support the hypothesis. Using a novel monoclonal antibody specific to GAβ, it was confirmed that GAβ is endogenously generated in the brain. Regarding the role of gangliosides in the facilitation of Aβ assembly, it has recently been reported that region-specific deposition of hereditary variant-type Aβs is determined by local gangliosides in the brain. Furthermore, it is likely that risk factors for AD, including aging and the expression of apolipoprotein E4, alter GM1 distribution on the neuronal surface, leading to GAβ generation.

Keywords: Alzheimer's disease; Amyloid β-protein; Seed; Ganglioside; Cholesterol

Contents

1. Introduction

An invariable and fundamental process in the pathogenesis of Alzheimer's disease (AD) is the assembly and deposition of the amyloid β-protein (Aβ). Aβ naturally assembles at high micromolarity concentrations *in vitro*. However, the concentration is very low (on the low nanomolarity order) in biological fluids, including the cerebrospinal fluid [1–3]. In the case of familial AD, the expression of responsible genes likely accelerates Aβ assembly owing to an enhanced Aβ generation [4]; however, no evidence has yet been provided suggesting that the level of Aβ generation increases in sporadic AD, the major form of the disease. Thus, it remains to be determined how Aβ

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starts to assemble *in vivo* in an age-dependent manner and in a region-specific manner.

Several mutations within the Aβ sequence are responsible for familial AD and hereditary amyloid angiopathy [5–7]. Notably, some of these mutations facilitate Aβ pathological processes without enhancing Aβ generation. The Arctic-type mutation even decreases the Aβ1–42/1–40 ratio in secreted Aβs, an increase in which is supposed to be one of the critical factors for Aβ assembly. Furthermore, these mutations also induce Aβ assembly and deposition that preferentially occur in a specific area in the brain. For example, the Dutch-type mutation (E693Q) induces Aβ deposition preferentially in the cerebral blood vessel walls, whereas the Arctic-type mutation (E693G) induces predominant Aβ deposition in the parenchyma of the cerebral cortex.

This line of evidence indicates that Aβ assembly in the brain is accelerated not only by the enhancement of Aβ generation, but also by as yet unclarified local assembly-promoting factors. To determine how Aβ starts to assemble and deposit in the brain, cerebral cortices of AD patients and nondemented aged individuals were previously examined by fractionation and immunochemical Aβ detection in the obtained fractions. In that study, a unique Aβ species was identified, which is characterized by its binding to the GM1 ganglioside (GM1), in a brain showing the early pathological changes of AD [8]. On the basis of the molecular characteristics of GAβ, it was hypothesized that GM1-bound Aβ (GAβ) acts as a seed for Aβ assembly in the AD brain. This chapter will focus on the molecular characteristics of GM1-bound Aβ (GAβ) and its pathological implications in AD.

2. GAβ in AD brain

GAβ was identified in the membrane fractions prepared from cerebral cortices of brains that showed the early pathological changes of AD, including diffuse plaque formation [8]. GAβ showed unique molecular characteristics as follows. First, this Aβ species showed a retarded mobility and a smeared appearance on SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Second, it was not detected using antibodies specific to the midportion of Aβ. Notably, these unique molecular characteristics of this Aβ species completely disappeared following treatment with methanol prior to SDS-PAGE. This line of evidence led us to speculate that this Aβ species is bound to a ganglioside. This possibility was strongly supported by the observation that this Aβ species was specifically recognized by the HRP-conjugated cholera toxin (CTX), which is a natural and specific ligand for GM1, one of the major gangliosides in the brain. Thus, it was concluded that this novel Aβ species is GM1-bound. Interestingly, GAβ shows a strong tendency to form large Aβ assemblies and has altered immunoreactivity. On the basis of these characteristics, it was hypothesized that Aβ adopts an altered conformation by binding to GM1 and acts as a seed for the assembly of soluble Aβ in the AD brain. Subsequently, several investigators performed *in vitro* studies and their results support the hypothesis [9–16]. In particular, Matsuzaki and his colleagues reported that the binding of Aβ to GM1 is facilitated in cholesterol-rich environment, and it is dependent on cholesterol-induced GM1 clustering in host membranes [15].

3. Facilitation of Aβ assembly in presence of GM1

The seeding activity of GAβ was examined using seed-free Aβ solutions, which were carefully prepared as previously reported [17]. Briefly, a synthetic Aβ peptide (Aβ1–40 or Aβ1–42) was dissolved in 0.02% ammonia solution, and undissolved peptides, which can act as preexisting seeds, were

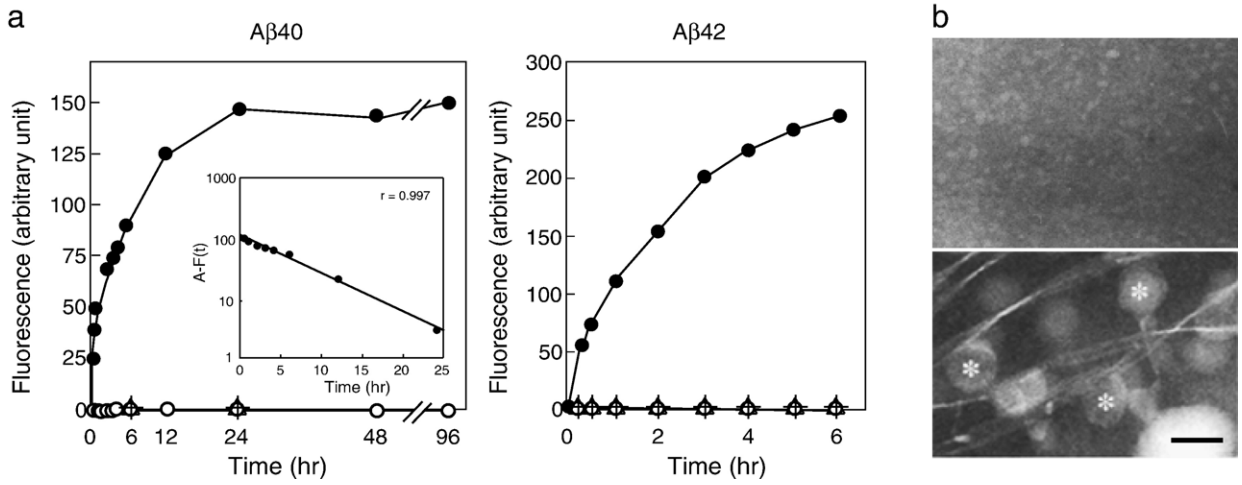

Fig. 1. Aβ fibrillogenesis in the presence of GM1. (a) Kinetics of Aβ fibrillogenesis. Solutions of synthetic Aβ (Aβ40 and Aβ42), after removal of undissolved peptide aggregates, were incubated at 50 μM and 37 °C in the presence of GM1-containing liposomes (filled circle) or GM1-lacking liposomes (plus), or incubated in the absence of liposomes (open circle). The GM1-containing liposomes were also incubated in the absence of Aβ (triangle). Thioflavin T (ThT) fluorescence intensity was obtained by excluding background activity at 0 h. Inset: Semilogarithmic plot of the difference, $A-F(t)$, versus incubation time (0–24 h). $F(t)$ represents the increase in fluorescence intensity as a function of time in the case of Aβ incubated with GM1-containing liposomes and A is tentatively determined as F (infinity). Linear regression and correlation coefficient values were obtained ($r=0.997$). $F(t)$ is described by a differential equation: $F'(t)=B-CF(t)$. (b) Electron micrographs of formed amyloid fibrils from soluble Aβ in the presence of GM1. Aβ40 solutions incubated at 50 μM and 37 °C for 24 h with GM1-containing liposomes (lower panel) or without liposomes (upper panel). The liposomes are indicated by asterisks. Bar, 50 nm. (Copyright 2004 by the Society for Neuroscience).

removed by ultracentrifugation. A seed-free Aβ solution showed no increase in the fluorescence intensity of thioflavin T (ThT), which specifically recognizes amyloid structures. In contrast, the addition of GM1-containing liposomes induced a marked increase in ThT fluorescence intensity [18] (Fig. 1a). Electron microscopy also revealed that Aβ assembly into amyloid fibrils in seed-free Aβ solutions specifically occurred in the presence of GM1-containing liposomes [18] (Fig. 1b).

4. Antibody specific to GAβ

To further characterize the GM1-induced facilitation of Aβ assembly and the molecular characteristics of GAβ, GAβ-specific antibodies were generated. To raise monoclonal antibodies, a membrane fraction from the cerebral cortices of subjects with abundant diffuse plaques was prepared, and GAβ was then purified from the membrane fraction by Prepcell electrophoretic fractionation. The purified GAβ was added to cultured spleen cells obtained from mice using an *in vitro* immunization technique. An IgM monoclonal antibody (4396) was initially obtained [19], and then an IgG monoclonal antibody (4396C) was generated using the genetic class-switch technique [18]. The specificity of 4396C binding to GAβ was confirmed by immunoelectron microscopy, a quantitative binding assay, and a dot blot analysis of liposomes carrying GAβ on their surfaces [18] (Fig. 2a–c). Notably, 4396C did not react with the free form of Aβ or GM1. It was examined whether GAβ acts as a seed using a novel antibody. In a kinetic study of fibril formation from soluble Aβ, the fluorescence intensity of ThT, which specifically recognizes amyloid structures, significantly decreased in the presence of 4396C in a dose-dependent manner [18] (Fig. 3). On the basis of these results, it was concluded that soluble Aβ binds to GM1, leading to the generation of GAβ, and then, this generated GAβ accelerates Aβ fibrillogenesis from soluble Aβ by acting as a seed.

5. Evidence of GAβ generation in brain

It was attempted to confirm GAβ generation in the brain using an antibody specific to GAβ. No immunoreactivity with 4396C was found in sections of AD brains fixed in

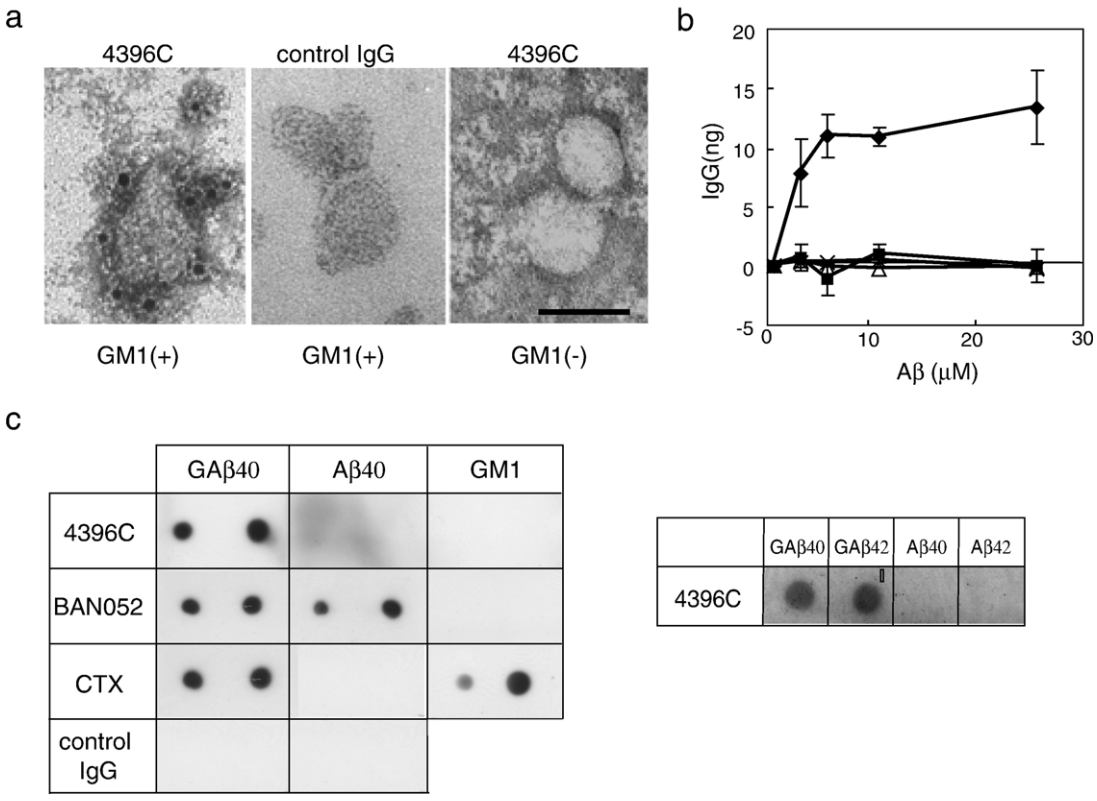

Fig. 2. Characterization of binding specificity of anti-GAβ antibody. (a) Immunoelectron micrographs of liposomes. GM1-containing and GM1-lacking liposomes were analyzed by immunoelectron microscopy of 4396C or isotype-matched control IgG staining following incubation with soluble Aβ40. GM1(+), GM1-containing liposomes; GM1(−), GM1-lacking liposomes. Bar, 50 nm. (b) Quantitative binding assay of 4396C to liposomes. GM1-containing liposomes were incubated with 4396C (diamond) or isotype-matched control IgG (filled square) following their mixing with soluble Aβ40 at indicated concentrations. GM1-lacking liposomes were also incubated with 4396C (triangle) or isotype-matched control IgG (X). (c) Dot blot analysis. Left panel: liposomes carrying GAβ (GAβ40), Aβ40, and GM1 in amounts equal to those contained in blotted liposomes (300 ng and 600 ng of Aβ40; 2 μg and 4 μg of GM1), were blotted. The blots were reacted with 4396C, BAN052, HRP-conjugated cholera toxin subunit B (CTX), or isotype-matched control IgG. Right panel: liposomes carrying GAβ (GAβ40 and GAβ42), prepared using Aβ40 or Aβ42, Aβ40 and Aβ42 in amounts equal to those contained in GAβ40 and GAβ42 (600 ng of each peptide), were blotted. The blots were reacted with 4396C. (Copyright 2004 by the Society for Neuroscience).

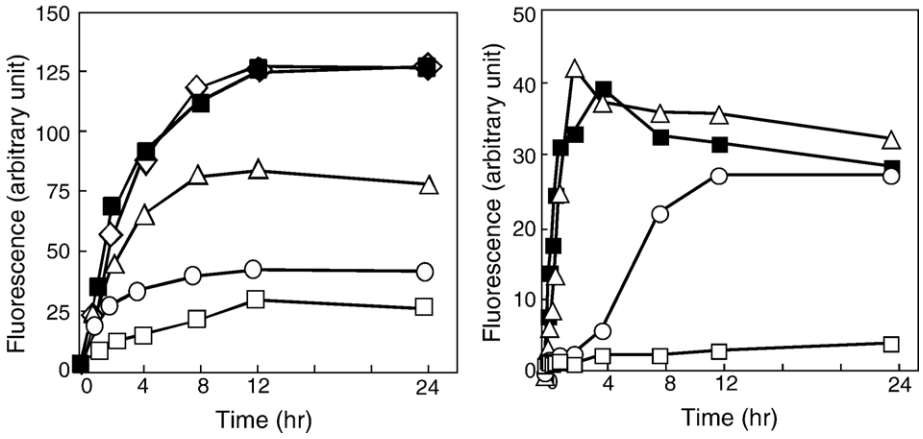

Fig. 3. Suppression of amyloid fibril formation from soluble Aβ40 by 4396C. Left panel: soluble Aβ40 was incubated with GM1-containing liposomes in the absence (filled square) or presence of an antibody (4396C or 4G8). The molar ratios of 4396C to soluble Aβ40 were 0.3:50 (triangle), 1.3:50 (circle) and 4:50 (open square), and that of 4G8 to Aβ40 was 4:50 (diamond). Right panel: soluble Aβ40 was incubated with preformed Aβ40 fibrils in the absence of an antibody (filled square) or in the presence of 4396C. The molar ratios of 4396C to soluble Aβ40 were 0.3:50 (triangle), 1.3:50 (circle) and 4:50 (open square). (Copyright 2004 by the Society for Neuroscience).

formaldehyde and pretreated with formic acid to enhance Aβ immunoreactivity; however, fixation with Kryofix, which eliminates the possibility of obtaining false-negative results that can occur in the case of fixation using formalin, made it possible to obtain intraneuronal staining with 4396C in the sections [18] (Fig. 4). To confirm the immunohistochemical detection of GAβ, fresh brain samples from nonhuman primates, which naturally develop Aβ deposition after age 25, were examined. The cerebral cortices of seven animals of different ages were examined. In the sections obtained from the aged animals (ages 30 and 36), a number of neurons were strongly immunostained by 4396C showing a granular pattern [18] (Fig. 5a). Double immunostaining of the sections of the aged animal revealed the colocalization of intraneuronal staining by 4396C with that of Aβ or GM1 [18] (Fig. 5b). Furthermore, GAβ immunoreacted with 4396C only in samples from the cerebral cortex of the aged animal [18] (Fig. 5c). These results indicate that GAβ is generated in the brain.

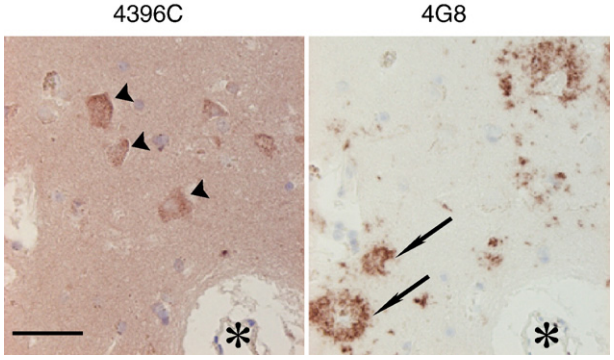

Fig. 4. Immunohistochemistry of GAβ in sections of AD brains. Immunostaining of serial sections of the cerebral cortex of an AD brain fixed in Kryofix and pretreated with SDS. Neurons (arrowheads) were immunostained by 4396C but not by 4G8, whereas plaques (arrows) were immunostained by 4G8 but not by 4396C. The asterisks indicate the same blood vessel in the serial sections. Bar, 50 μm. (Copyright 2004 by the Society for Neuroscience).

6. GM1 accumulation induced by AD risk factors: putative alteration of GM1 metabolism in AD

The next issue that should be addressed is how GAβ is generated in the brain. Aβ is physiologically secreted into the extraneuronal space and GM1 is expressed on neuronal surfaces. However, GAβ has not been detected in brains showing no Aβ deposition [8]. Matsuzaki and his colleagues previously attempted to elucidate the molecular mechanism underlying the binding of Aβ to GM1 using liposomes with various compositions of lipids. They found that the binding of Aβ to GM1 is accelerated in cholesterol-rich environments and that this is due to the formation of GM1 clusters, suggesting that the lipid composition of host membranes is a critical factor for the facilitation of GAβ generation [15]. It remains to be clarified whether the local contents of cholesterol and GM1 in neuronal membranes are altered under certain biological conditions, including aging and the expression of apolipoprotein E (apoE), which are strong risk factors for AD development. In this regard, a previous study showed that the asymmetric distribution of cholesterol throughout the lipid bilayers of synaptic plasma membranes (SPMs) changes with age, resulting in an approximately twofold increase in cholesterol content in the exofacial leaflet of SPMs prepared from old mice compared with that from young mice [20]. On the basis of this finding, cholesterol distribution in the SPM of human apoE knock-in mice was analyzed, and it was found that apoE4 knock-in mice showed an approximately twofold increase in cholesterol content in the exofacial leaflet compared with apoE3 knock-in mice and wild-type mice. Thus, it was investigated whether GM1 content in neuronal membranes can be altered under these conditions. Analysis of brains of apoE3 or apoE4 knock-in mice at three different ages (2 months, 1 year, and 2 years) revealed that GM1 content in detergent-resistant membrane microdomains (DRMs) obtained from synaptosomes increased with age, and that this increase was more marked in the

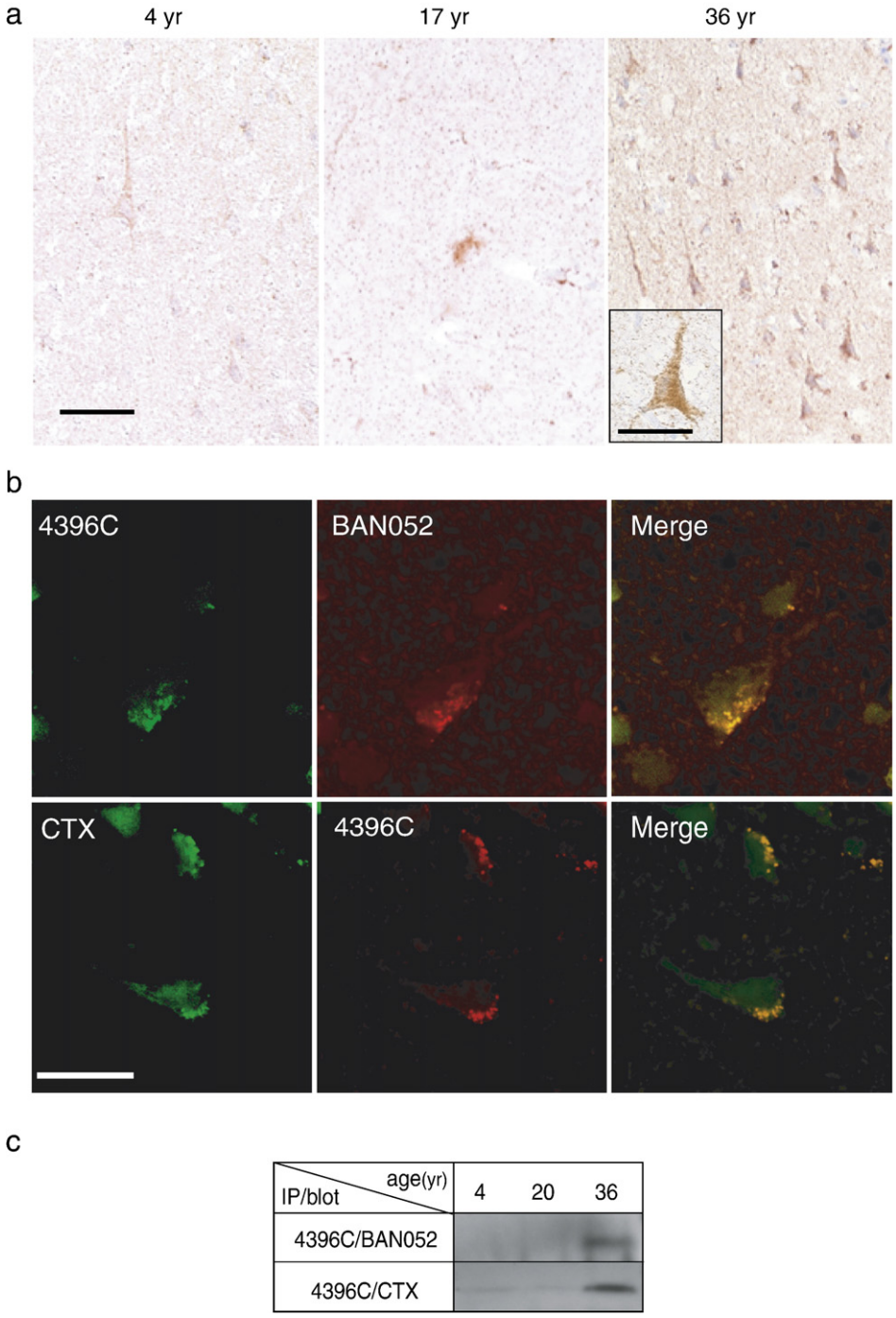

Fig. 5. Detection of GAβ in sections of nonhuman primate brains. (a) Immunostaining by 4396C of sections of the cerebral cortices of nonhuman primate brains, which were fixed in paraformaldehyde, from animals of different ages. Bar, 50 μm. Inset: higher magnification. Bar, 20 μm. (b) Double immunostaining of sections of the cerebral cortex of a 36-year-old primate brain, which was fixed in paraformaldehyde, following the blocking of autofluorescence by pretreatment with Sudan Black B. Immunostaining by 4396C colocalizing with that by BAN052 or CTX is shown in the merged image. Bar, 25 μm. (c) Immunoprecipitation of GAβ by 4396C from cerebral cortices of primates of different ages. Immunoprecipitates were blotted and reacted with BAN052 or HRP-conjugated cholera toxin subunit B (CTX). (Copyright 2004 by the Society for Neuroscience).

apoE4 knock-in mouse brains than in the apoE3 knock-in mouse brains [21] (Fig. 6). Interestingly, the DRMs, which showed an age-dependent increase in GM1 content, were apparently unique microdomains distinct from lipid rafts. Presently, it remains to be clarified how GM1 is accumulated in SPMs in an age-dependent manner and an apoE4-expression-dependent manner; however, another intriguing issue is whether an increase in GM1 content in such microdomains is sufficient to induce fibrillogenesis from soluble Aβ. To clarify this issue, soluble Aβ was incubated in the presence of synaptosomes obtained from aged apoE4 knock-in mouse brains. As shown in Fig. 7, the assembly of

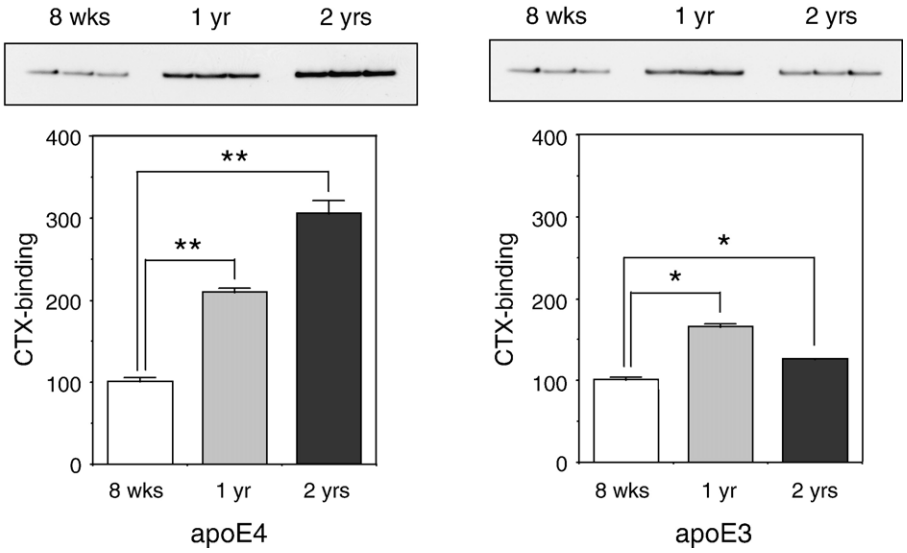

Fig. 6. GM1 levels in the DRM fractions isolated from brain synaptosomes. DRM fractions were prepared from three different age groups of apoE3- and apoE4-knock-in mice. Each lane contains 0.1 μg protein of the DRM fraction isolated from synaptosomes prepared from five brains combined. The blots were 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 ± S.D. of three values $^{**}p<0.0001$, $^{*}p<0.001$ (One-way ANOVA combined with Sheffe's test). (From Yamamoto et al., FEBS Lett 569: 135–139, 2004).

soluble Aβ into amyloid fibrils was significantly accelerated in the presence of such synaptosomes [21]. This finding suggests that a change in the lipid composition in neuronal membranes, particularly in DRMs, underlie the facilitation of Aβ assembly and deposition through GAβ formation. This has recently been supported by the following studies. First, Fredman and her colleagues found that the GM1 content is high in membrane microdomains isolated from the frontal cortex, but not in those isolated from the temporal cortex, reflecting the earlier and later stages of AD development,

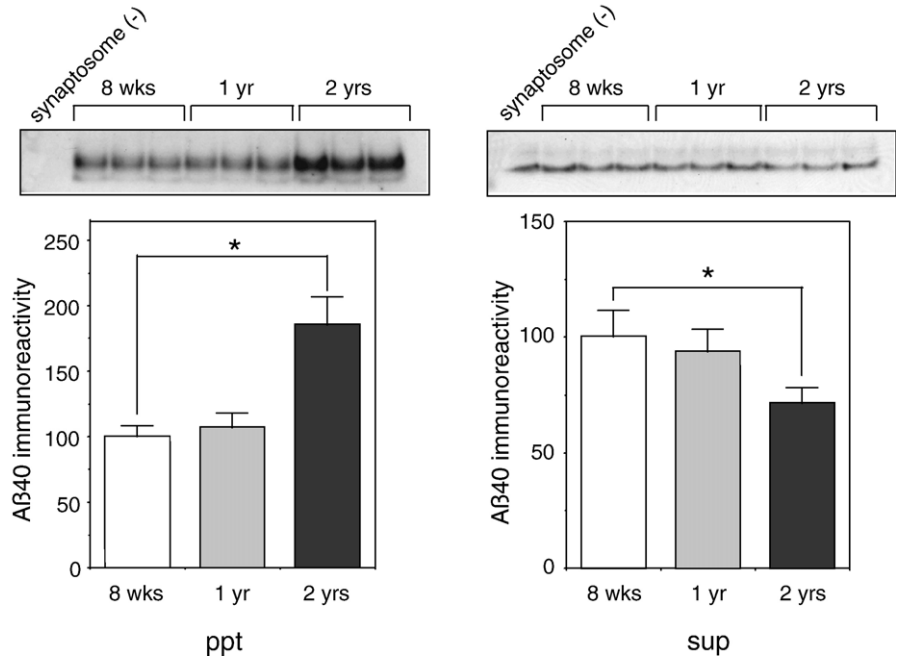

Fig. 7. Acceleration of Aβ assembly in the presence of synaptosomes prepared from aged apoE4-knock-in mouse brains. Synaptosomes were isolated from three different age groups of apoE4-knock-in mice. Aβ solutions were incubated at 50 μM and 37 °C for 24 h in the presence or absence of the synaptosomes. Western blots of precipitates (ppt) and supernatants (sup) of the incubation mixtures. The precipitates from 100 μl of incubation mixture were subjected to SDS-PAGE following solubilization of assembled Aβ in formic acid. 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 weeks old mice are indicated. Each column represents the average ± S.D. of three values. $^{**}p<0.0001$, $^{*}p<0.05$ (One-way ANOVA combined with Sheffe's test). (From Yamamoto et al., FEBS Lett 569: 135–139, 2004).

respectively [22]. Second, Cole and his colleagues reported that GM1 level increases in amyloid-positive neuritic terminals obtained from the AD cortex [23].

7. Region-specific Aβ assembly and deposition induced by local gangliosides

Several mutations within the Aβ sequence are responsible for familial AD and hereditary amyloid angiopathy. Notably, these mutations induce Aβ assembly and deposition that preferentially occur in a specific area in the brain. For example, the Dutch-, Italian- and Iowa-type mutations induce Aβ deposition preferentially in cerebral blood vessel walls [5,24–26], whereas the Arctic-type mutation (E693G) induces predominant Aβ deposition in the parenchyma of the cerebral cortex [7]. The Flemish-type mutation induces Aβ deposition that starts from the outer surface of cerebral blood vessels [27]. This region-specific deposition led us to hypothesize that the assembly and deposition of these hereditary variant-type Aβs are dependent on the presence of local factors, for example, the presence of region-specific gangliosides. To examine this possibility, lysates of human

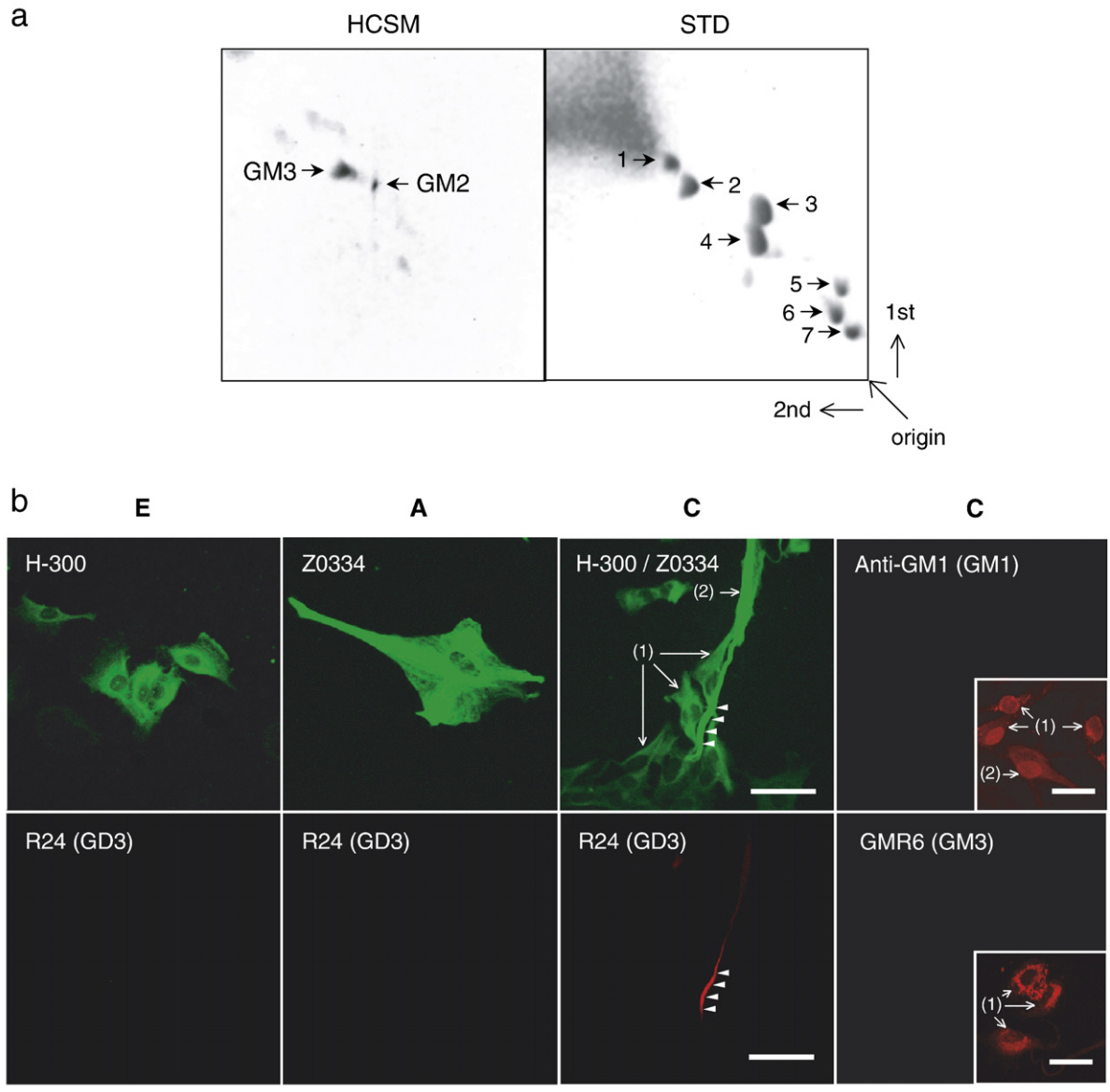

Fig. 8. Selective expression of GM3 and GD3 by cerebrovascular smooth muscle (HCSM) cells and astrocytes, respectively. (a) Gangliosides extracted from HCSM cells were separated by two-dimensional TLC. STD: standard. Arrows 1, 2, 3, 4, 5, 6 and 7 indicate GM3, GM2, GM1, GD1a, GD1b, GT1b and GQ1b, respectively. (b) Immunocytochemistry of cultured endothelial cells and astrocytes and coculture of these cells. Endothelial cells and astrocytes were immunostained with antibodies against CD31 (H-300) and GFAP (Z0334), respectively. The cocultured endothelial cells and astrocytes were immunostained with both H-300 and Z0334. The cells were also double-immunostained with an antibody against GD3 (R24). The cocultured cells were also reacted with anti-GM1 or GMR6 with (insets) or without pretreatment for permeabilization. Arrows (1) and (2) indicate endothelial cells and astrocytes, respectively. Arrowheads indicate the process of astrocyte. Bars, 50 μm. (From Yamamoto et al., FEBS Lett 579: 2185–2190, 2005).

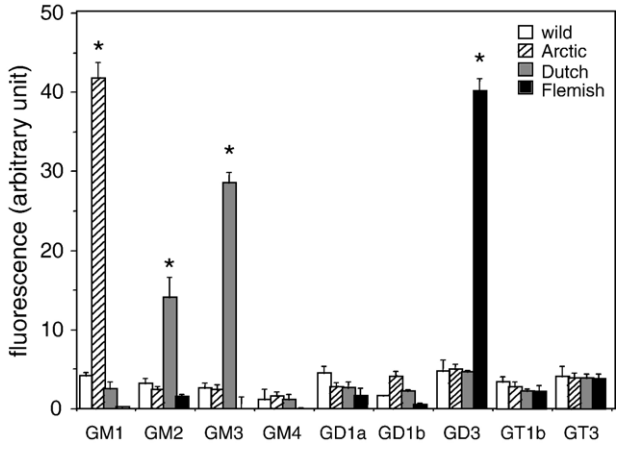

Fig. 9. Assembly of hereditary variant types of Aβ in the presence of various gangliosides. ThT fluorescence intensities of Aβ solutions incubated in the presence of various gangliosides. The ThT fluorescence intensities of solutions containing Aβ alone or liposomes alone were negligible (data not shown). Each column indicates the average ± SD of four values. *$p < 0.0001$ versus the value of the wild-type Aβ (one-way ANOVA combined with Sheffe's test). (From Yamamoto et al., FEBS Lett 579: 2185–2190, 2005).

cerebrovascular smooth muscle (HCSM) cells, which are a preferable site for the deposition of these Aβs, were subjected to thin-layer chromatography to determine the gangliosides that are expressed by these cells. Notably, HCSM cells exclusively express GM3 [28] (Fig. 8a). In contrast, GD3 was preferentially expressed by astrocytes that were in direct contact with endothelial cells [28] (Fig. 8b). Taken together with the finding that the assembly of Dutch- and Flemish-type Aβs is accelerated in the presence of GM3 and GD3, respectively [28] (Fig. 9), these results suggest that the region-specific assembly and deposition of Aβ depend on local gangliosides in the brain. In contrast to the expression of GM3 and GD3, which are likely responsible for the region-specific deposition of particular Aβ species, GM1 is rather broadly expressed in the brain, at least, apparently beyond the preferential site of Aβ deposition. As aforementioned, GM1 content in membrane microdomains of neuronal membranes can be altered under certain biological conditions such as aging and the expression of apoE4. Thus, it will be critically important to elucidate whether GM1 expression changes in a region-specific manner in the brain before AD development.

8. Conclusion

Previous studies suggest that the expression of gangliosides is an environmental factor that induces the assembly and deposition of Aβ in the brain. A challenge for future studies is to elucidate the mechanism underlying GAβ generation in the brain, which may provide new insights into the pathophysiology of AD. Moreover, on the basis of the finding that the conformation of GAβ is distinct from those of soluble Aβs and Aβs assembled into amyloid fibrils, it may be possible to develop a novel therapeutic strategy that specifically targets GAβ as a seed for Aβ assembly.

Acknowledgments

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