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The 5XFAD Mouse Model of Alzheimer's Disease Exhibits an Age-Dependent Increase in Anti-Ceramide IgG and Exogenous Administration of Ceramide Further Increases Anti-Ceramide Titers and Amyloid Plaque Burden

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Abstract

We present evidence that 5XFAD Alzheimer's disease model mice develop an age-dependent increase in antibodies against ceramide, suggesting involvement of autoimmunity against ceramide in Alzheimer's disease pathology. To test this, we increased serum anti-ceramide IgG (2-fold) by ceramide administration and analyzed amyloid plaque formation in 5XFAD mice. There were no differences in soluble or total amyloid- β levels. However, females receiving ceramide had increased plaque burden (number, area, and size) compared to controls. Ceramide-treated mice showed an increase of serum exosomes (up to 3-fold using Alix as marker), suggesting that systemic anti-ceramide IgG and exosome levels are correlated with enhanced plaque formation.

Keywords

Alzheimer's disease; amyloid; antibodies; ceramide; exosomes; mice

INTRODUCTION

Alzheimer's disease (AD) is a neurodegenerative disease that affects cognition and behavior [1] with only temporary palliative therapies available [2, 3]. While the cause of sporadic AD is unclear, the leading hypothesis is accumulation of amyloid- β (A β) peptides initiating a cascade of events leading to altered synaptic transmission and neuronal death [4, 5]. Evidence comes from genetic studies identifying mutations in genes such as amyloid- β protein precursor (A β PP) [6] and presenilin-1 [7]. Individuals with these mutations have increased A β production and develop early-onset AD [1]. The majority of therapeutic research has focused on targeting A β peptides and aggregates directly or their synthesis and clearance [2].

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Ceramide is a membrane sphingolipid involved in cell signaling [8, 9] that is elevated in AD [10–13] and animal models [14–16]. Ceramide increases the half-life of β -site amyloid- β protein precursor cleaving enzyme 1, which increases A β production [17]. Ceramide generation by hydrolysis of sphingomyelin via neutral sphingomyelinase is required during amyloid-induced cell death in neural and endothelial cells [18–22], and inhibition of ceramide-generating enzymes in AD model mice reduces A β burden [15, 23]. Additionally, elevated serum ceramide may be a risk-factor for AD [24]. Ceramide is also enriched in secreted extracellular vesicles known as exosomes [21, 25], which may play a role in the progression of AD [26] or aid in A β clearance [27]. We have previously reported that A β peptides stimulate exosome secretion from astrocytes *in vitro* [21], which suggests that exosome levels may be elevated during AD. In this study, we examined a novel approach for AD treatment by administering ceramide to increase serum anti-ceramide antibodies in 5XFAD mice and hypothesized that this would lower A β levels and serum exosome levels.

METHODS

Animals and ceramide administration

Animal experiments were approved by Georgia Regents University's Institutional Animal Care and Use Committee. Mice expressing five mutations in human A β PP and PS1 (5XFAD) (B6SJL-Tg[A β PP *K670N*M671L*I716V*V717I, PSEN1*M146*L286V]6799Vas/J) under the Thy1 promoter were purchased from The Jackson Laboratory. 5XFAD mice are robust in their A β ₄₂ production with visible plaques at 2 months [28]. We administered 25 μ g of C18:0-ceramide in two locations subcutaneously (3 doses, 50 μ L each) at 2-week intervals to hemizygous 5XFAD mice, initially 10-weeks-old (5 males, 5 females). The initial emulsion was prepared with Complete Freund's Adjuvant and PBS 1:1, and booster doses were prepared with Incomplete Freund's Adjuvant. Mice were decapitated 9 days following the third dose. No behavioral or health problems were observed.

Sample preparation and analysis

One hemi-brain was frozen at -80°C , and the other was fixed in 4% p-formaldehyde/PBS for cryosectioning. Hemi-brains were homogenized in cold NaCl (50 mM, 1 mL/100 mg tissue) with protease inhibitors. To harvest exosomes, sera (50 μ L diluted to 5.2 mL PBS) were centrifuged at $20,000 \times g$ for 30-min followed by ultracentrifugation at $110,000 \times g$ at 4°C for 2 h. Exosomes were resuspended in 150 μ L SDS buffer for western analysis. The membrane was blocked with 5% non-fat milk and probed using anti-Alix (Santa Cruz, 1A12, 1:1000 dilution) overnight followed by HRP-conjugated anti-mouse IgG (Jackson, 1:5000 dilution; also used for anti-ceramide ELISA) and ECL detection substrate.

To extract soluble A β from brains, diethylamine was added to a final concentration of 0.2%, and the samples were centrifuged at $100,000 \times g$ for 1 h at 4°C . 100 μ L of 0.5M Tris (pH 6.8) were added to 1 mL supernatant to neutralize diethylamine, and samples were diluted for ELISA. To extract total A β , 200 μ L of homogenate were added to 440 μ L cold formic acid (88%), and samples were sonicated for 1 min on ice and centrifuged at $150,000 \times g$ for

1 h at 4°C. 100 μ L of supernatant were diluted into 2 mL of neutralization solution (1M Tris base, 0.5 M Na₂HPO₄) and diluted for ELISA.

A β ₄₂ ELISA was performed with 50 μ L diluted sample strictly according to the manufacturer's instructions (Life Technologies). Serum anti-ceramide titers from wildtype (C57JB1/6) and 5XFAD mice were determined by ELISA as described [29] using Immulon-1B plates coated with ceramide or sphingomyelin in 100% ethanol. Ethanol-only controls were subtracted as background from coated wells.

To label plaques, cryosections were washed for 1 min each in 70% and 80% ethanol and incubated with 1% thioflavin S in 80% ethanol for 15 min. Slides were washed for 1 min each in 80% and 70% ethanol, rinsed with deionized water, and mounted [30].

Statistical analysis

Brain sections were imaged by epifluorescence microscopy. Cortical images (4–5 each) were acquired from 3 coronal sections per animal (bregma: –1.25 to –1.75), background subtracted, and analyzed with ImageJ for plaque number, total plaque area, and average plaque area. Plaques in different sections from one animal were uniform at the locations examined. Densitometry was performed using ImageJ. Anti-ceramide IgG titers were analyzed by one-way (Fig. 1A, G) or two-way (Fig. 1B–F) ANOVA with Bonferroni *post-hoc* test. All other data, were analyzed by unpaired *t*-test with Welch's correction. Results showing $p < 0.05$ were considered to be statistically significant.

RESULTS

Initial ELISA experiments showed elevated anti-ceramide in 5XFAD mouse sera over wildtype. To confirm specificity, we coated plates with varying amounts of C18:0-ceramide and found increased IgG-binding with increasing amount of ceramide (Fig. 1A). To rule out binding due to non-specific hydrophobic interactions, we performed ELISA with C18:0-sphingomyelin, similar in structure to ceramide (Fig. 1I). We observed no significant binding to sphingomyelin (Fig. 1B). We evaluated wildtype and 5XFAD mouse sera at different ages for anti-ceramide activity and found significant genotype- and age-dependent increases (two-way ANOVA, $p < 0.001$) using C18:0-, C24:1-, and C16:0-ceramides (Fig. 1C–E). Serum IgGs showed higher reactivity (2–2.5-fold) against C18:0- and C24:1-ceramides compared to C16:0-ceramide. However, there were no differences between males and females in different age groups (Fig. 1F and not shown). We used rabbit anti-ceramide IgG [29] as a positive control.

Untreated 5XFAD mice had between 2- to 3.5-fold higher titers of anti-ceramide than wildtype (Fig. 1C–E, G), suggesting that accumulating A β indirectly stimulates an immune response against ceramide. We were interested in further increasing titers by administering ceramide to 5XFAD mice. Ceramide administration increased anti-ceramide titers 2-fold (Fig. 1G). One possible explanation of increased anti-ceramide titers could be increased circulating exosomes, which are ceramide-enriched [21, 25]. Using the marker Alix to estimate relative abundance of exosomes [21, 23, 27], we found a 2.4-fold increase in serum exosomes from treated versus non-treated 5XFAD mice (Fig. 1H). No statistical differences

between the control and ceramide-treated mice were noted for the amount of soluble or total brain A β ₄₂. However, females displayed a larger total A β ₄₂ burden than males (Fig. 2A–F). Female 5XFAD mice receiving ceramide showed an increase in cortical plaque number, area, and size (Fig. 2I, L, O) over controls. Males showed an increase in plaque number but had slightly smaller plaques in the ceramide-treated group (Fig. 2H, N).

DISCUSSION

We previously reported that astrocytes increased ceramide levels and release of exosomes *in vitro* after exposure to A β _{25–35} [21], and recent work suggests a role for exosomes in A β aggregation and clearance [23, 27]. It is well known that antibodies can affect brain tissue despite the blood-brain barrier. A prominent example is demyelination in multiple sclerosis, which involves invasion of white blood cells through the blood-brain barrier into brain tissue and secretion of antibodies that degenerate myelin [31]. Two recent reports show that passive immunization of AD-model mice against ApoE before [32] and after plaque accumulation [33] resulted in reduced A β deposition, microglial response, and improvement in behavioral deficits. Therefore, we administered ceramide to 5XFAD mice with the hypothesis that increased anti-ceramide [29] would aid the exosome-dependent clearance of A β [27] as rabbit anti-ceramide IgG [29] prevented exosome-induced aggregation of A β ₄₂ [23]. However, we found no significant differences in terms of A β ₄₂ levels, but ceramide-treated females had a 33% increase in the mean total A β ₄₂ level (Fig. 2F) and increased plaque load (Fig. 2I, L, O). It is well-known that females have higher incidence of AD [34, 35], and we have previously shown that 5XFAD female mice have higher A β ₄₂ and plaque levels [23]. While the mechanism of gender-dependent accumulation of A β is unclear, AD-related mouse studies have shown increased accumulation of 2-hydroxyceramides [36] and C16:0- and C18:0-ceramides as well as enzymes in ceramide biosynthesis [11] in females. It is possible that increased ceramide accumulation in females further exacerbates amyloidogenic processing of A β PP [17, 39].

Serum exosome content was elevated 2.4-fold in ceramide-treated mice compared to untreated controls. One interpretation is that increased exosomes contribute to plaque deposition rather than A β ₄₂ clearance with the assumption that serum exosome content reflects brain parenchyma exosome content, although this has not been shown. Previous data with A β -stimulated astrocytes [21] suggest that exosome secretion is increased during AD and in animal models, which is likely given the elevation of brain ceramides [10–16]. It is also possible that anti-ceramide antibodies could prevent exosome clearance ultimately through the urine, allowing their accumulation in the blood. Alternatively, it is possible that increased exosome secretion in AD may trigger production of anti-ceramide antibodies shown here in 5XFAD mice. Interestingly, the highest serum IgG reactivity was against C18:0- and C24:1-ceramides, which we previously reported as being enriched in exosomes from A β -stimulated astrocytes [21]. Future studies will examine anti-ceramide in human AD and whether this phenomenon is mediated by an increase in circulating ceramide-enriched exosomes.

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Fig. 1.

Age-dependent increase in serum anti-ceramide titers in 5XFAD mice and further increase in titers and serum exosomes following ceramide administration. A–G) Graphs showing relative antibody titers against the indicated sphingolipid (500 ng, B–G). Data are presented as mean $\pm$ SEM of the O.D. $\times$ dilution factor (y-axis) following subtraction of background values using an uncoated (ethanol solvent) control for each sample against either (A) amount of ceramide coating, (B–E) indicated genotype/age (months), (F) sex/age (5XFAD only), or (G) genotype/treatment (all mice 16 weeks). Statistically significant differences are denoted by groups with unshared labels (A, $p < 0.05$) or indicated by brackets and p -values and (B–G). Rabbit anti-ceramide IgG was included as a positive control (B–E). H) Immunoblot for Alix in exosomes isolated from sera of 5XFAD mice and 5XFAD mice treated with ceramide (5X+Cer) as an indicator of relative exosome amount. The graph below shows densitometry analysis of Alix bands ($n = 6$, mean $\pm$ SEM arbitrary units). I) Chemical structures of C18:0 sphingomyelin, C18:0 ceramide, and C24:1 ceramide used to determine relative antibody titers.

Fig. 2.

Ceramide administration to 5XFAD does not alter mean amyloid levels but increases plaque burden in females. A-F) Dot plots showing soluble (A-C) and total (D-F) amyloid levels in control (5XFAD) and ceramide-treated (5X+Cer) mice determined by Aβ₄₂ ELISA for both sexes (A,D, *n* = 6), males (B,E, *n* = 3) and females (C,F, *n* = 3). G-O) Histochemical plaque data from thioflavin S-stained brain sections of control mice (5XFAD) or ceramide-treated mice (5X+Cer). Plaques per image field (G-I), total plaque area (J-L), and area per plaque (M-O) were analyzed by ImageJ for both sexes (G,J,M, *n* = 6), males (H,K,N, *n* = 3), and females (I,L,O, *n* = 3). All data represent mean±SEM. *P*-values are indicated when statistically significant differences were found.