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Progressive inflammatory pathology in the retina of aluminum-fed 5xFAD transgenic mice

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Abstract

At least 57 murine transgenic models for Alzheimer's disease (Tg-AD) have been developed to overexpress the 42 amino acid amyloid-beta (A β 42) peptide in the central nervous system (CNS). These '*humanized murine Tg-AD models*' have greatly expanded our understanding of the contribution of A β 42 peptide-mediated pro-inflammatory neuropathology to the AD process. A number of independent laboratories using different amyloid-overexpressing Tg-AD models have shown that supplementation of murine Tg-AD diets and/or drinking water with aluminum significantly enhances A β 42 peptide-mediated inflammatory pathology and AD-type cognitive change compared to animals receiving control diets. In humans AD-type pathology appears to originate in the limbic system and progressively spreads into primary processing and sensory regions such as the retina. In these studies, for the first time, we assess the propagation of A β 42 and inflammatory signals into the retina of 5xFAD Tg-AD amyloid-overexpressing mice whose diets were supplemented with aluminum. The two most interesting findings were (1) that similar to other Tg-AD models, there was a significantly accelerated development of A β 42 and inflammatory pathology in 5xFAD Tg-AD mice fed aluminum; and (2) in aluminum-supplemented animals, markers for inflammatory pathology appeared in both the brain and the retina as evidenced by an evolving presence of A β 42 peptides, and accompanied by inflammatory markers — cyclooxygenase-2 (COX-2) and C-reactive protein (CRP). The results indicate that in the 5xFAD Tg-AD model aluminum not only enhances an A β 42-mediated inflammatory degeneration of the brain but also appears to induce AD-type pathology in an anatomically-linked primary sensory area that involves vision.

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1. Introduction

The 5xFAD murine Tg-AD model, containing 5 human transgenes carrying mutations in the beta-amyloid precursor protein (β APP) and presenilin-1 (PSEN-1): [APP KM670/671NL (Swedish), APP I716V (Florida), APP V717I (London), PSEN1 M146L (A>C), PSEN1 L286V; currently one of the more advanced Tg-AD models], rapidly develops severe amyloid pathology accumulating high levels of neurotoxic 42 amino acid amyloid beta ($A\beta$ 42) peptide by ~1.5 months first in the murine brain cortex [1–3; unpublished observations]. Out of multiple Tg-AD models studied by our laboratory (Tg2576, PSAPP, 3xTg-AD, etc.) the 5xFAD model exhibits the most rapid and prolific accumulation of $A\beta$ 42 peptides in the brain and retina of any Tg-AD animal studied to date [2–4]. Whether amyloidogenesis originates independently in the brain and retina, or whether amyloidogenic signals originate in the brain and spread via the primary visual cortex and thalamus to the retina is open to question, but current data now favor the latter possibility [4–8]. Several laboratories have independently studied the effects of supplementing the diets of amyloid-overexpressing Tg-AD murine models (Tg-2576, APP/PS1, 5xFAD, etc.) with aluminum (as chloride, lactate, maltolate or sulfate) and have evaluated the impact of ingested aluminum with pathological outcome [9–17]. The general consensus is that the presence of aluminum in the diets of Tg-AD murine models enhances AD-type neuropathology: (1) by up-regulating the abundance of pro-inflammatory microRNAs [3], (2) by progressively exacerbating the incidence of oxidative stress [9–12]; (3) by hastening the appearance of $A\beta$ 42 peptides, accelerating $A\beta$ 42 oligomerization, amyloidogenesis and senile plaque deposition [11–13]; and (4) by increasing apoptosis in brain cells that temporally correlate to deficits in memory, cognition and spatial learning [14–17], all of which are features characteristic of AD neuropathology [9–17]. In these experiments we studied the evolution of $A\beta$ 40 and $A\beta$ 42 peptides, and the AD-relevant pro-inflammatory biomarkers cyclooxygenase-2 (COX-2) and C-reactive protein (CRP) in the brain and retina of 5xFAD Tg-AD mice fed aluminum in their diet and age-matched controls. The results indicate that in aged 5xFAD mice $A\beta$ 40 and $A\beta$ 42 peptides and COX-2 and CRP accumulate in the murine brain and retina, and for the first time link an aluminum-induced AD-type brain cortical pathology to the up-regulation of retinal pathology in an advanced 5xFAD Tg-AD model.

2. Methods

2.1. Reagents, brain and retinal tissues, protein extraction and quality control

Reagents used in these experiments were obtained from commercial suppliers and were used without further purification. Age-matched control and 5xFAD brain and/or retina at 0, 1.5, 3 and 5 months were analyzed for $A\beta$ 40 and $A\beta$ 42 peptide and COX-2 and CRP protein levels using ELISA and Western analysis [3,5–8]. Further details on the quality control for these 5xFAD brain and retinal samples, sample handling and analysis have been recently published [3,18–21]. Total brain and retinal RNA and protein were isolated using TRIzol reagent; RNA was archived for further analysis (Invitrogen, Carlsbad, CA, USA). Protein concentrations were determined using a dotMETRIC microassay as previously described (sensitivity 0.3 ng protein/ml; Chemicon-Millipore, Billerica, Massachusetts, USA) [18–26].

2.2. 5xFAD animals

5xFAD animals and controls (C57BL6/SJL founder strains) were purchased and reared according to the supplier's instructions (<http://jaxmice.jax.org/jaxnotes/507/507r.html>; Jackson Laboratories, Bar Harbor, MA, USA). A total of 13 female animals per group were randomized to receive either standard rodent chow (meal-ground pellets; Standard Laboratory rodent diet 5001; 'The Standard of Biomedical Research for Over 70 years'; http://www.labdiet.com/cs/groups/lolweb/@labdiet/documents/web_content/mdrf/mdi4/~edisp/ducm04_028021.pdf; LabDiet St. Louis, MO, USA), or a diet enriched in Al (as sulfate; commonly used as food additive and water purification agent; http://www.generalchemical.com/assets/pdf/Dry_Alum_Food_Grade_PDS.pdf; 100 mg Al/kg diet; LD₅₀ for aluminum sulfate in mice ~980 mg Al/kg). Food was provided ad libitum from birth; 5xFAD mice begin eating solid food at about 1 month of age; at 0, 1, 1.5, 3 and 5 months whole brain (cortex) and whole retinal tissues were obtained from the same animal for both protein and peptide analyses.

2.3. ELISA and Western analysis

Western gels and enzyme-linked immunoabsorbent assay (ELISA) were performed for quantification of A β 40 and A β 42 peptides and total A β 40 + A β 42 peptide load in control and transgenic mouse brain and retina as previously described [18–20,24–26]. Western immunoblots were performed using murine- and/or human-specific primary antibodies directed against A β 40 or A β 42 peptide (BDI350; ab20068 or ab10148; Abcam, Cambridge, MA, USA), the control protein marker β -actin (3598–100; Sigma-Aldrich Chemical Company, St. Louis, Missouri, USA), CRP (N-14; sc-18304), CRP (H-90; sc-30047) and/or CRP (C-13; sc-18306), COX-1 (AS70; sc-52757 and/or 41272; sc-70878), COX-2 (H3; sc-376861 and/or H-62; sc-7951; Santa Cruz Biotechnologies, CA, USA) using methods previously described by our group [18–27]. Both COX-2 and CRP levels were initially compared to an unchanging internal β -actin in the sample and then these values were used to derive COX-2 and CRP relative expression levels. Three different antibodies were used for CRP detection and COX-1 was used as the control for COX-2 as previously described [29].

2.4. Statistical analysis and data interpretation

All statistical procedures for ELISA and protein (Western) abundance were analyzed using a two-way factorial analysis of variance (p , ANOVA) using programs and procedures in the SAS language (Statistical Analysis Institute, Cary, NC, USA) and as previously described [27–29]. Only p -values of less than 0.05 (ANOVA) were considered to be statistically significant. Figures were generated using Excel 2008 (Microsoft Corporation, Redmond, WA), Adobe Illustrator CS3 ver 11.0 and Photoshop CS2 ver 9.0.2 (Adobe, San Jose, CA).

3. Results

A β 42 peptide, COX-2 and CRP abundances were quantified in 5xFAD brain cortex and whole retina in 5xFAD animals fed a control diet and from the brain and retina of age-matched 5xFAD animals fed a diet supplemented with aluminum (sulfate). Time points examined were 0, 1.5, 3 and 5 months; the first amyloid (A β peptide) pathology appears at about 1.5 months [1–3]. For every time point examined there were no differences in body

weight or general state of health between the control and aluminum-fed 5xFAD Tg-AD animals. At 1.5 months all animals showed accumulation of A β 40 and A β 42 peptide, COX-2 and CRP in 5xFAD brain and retina progressively accumulated with increasing age; these increases tapered off after 3 months and leveled off at the 5 mo time point (Table 1, Fig. 1). The data indicate that like in the brain, aged 5xFAD mice retina on control diets display an increased A β peptide load, an effect that was exacerbated in the retina of aluminum-fed animals. For example after 3 mo combined A β 40 + A β 42 peptide load in control diet fed 5xFAD animals increased to a mean of 140-fold in the brain and 91-fold in the retina, and in aluminum-fed animals these values increased to 177-fold and 115-fold in the brain and retina, respectively (Table 1). Similarly, COX-2 protein increased 3.1-fold and 4.2-fold in the retina and brain in 5xFAD animals receiving control diets, and increased, respectively 6.3-fold and 7.4-fold in the retina and the brain of 5xFAD animals receiving aluminum supplemented diets. CRP protein was found to increase 5.2-fold and 7.1-fold in the retina and brain of animals receiving control diets, and 15.5-and 13.7-fold in the retina and brain of animals receiving aluminum supplemented diets. Hence the inclusion of aluminum in the diet of 5xFAD mice more than doubled the abundance of the pro-inflammatory markers COX-2 and CRP in the brain and retina (Table 1). To further study the kinetics of COX-2 and CRP evolution in aging 5xFAD mouse retina, a time course of 0, 1.5, 3.0 and 5 months was chosen and whole retinas were analyzed (Fig. 1). Both brain–retina and the time course results indicate that aluminum in the diet increases A β 40 + A β 42 peptide load and COX-2 and CRP abundance in both the brain and retina as 5xFAD age and develop AD-type neuropathology. Interestingly up-regulation of A β peptides, COX-2 and CRP are known to accompany anesthesia-induced cognitive impairment and these biomarkers increase significantly in late-onset AD [24,26,29].

4. Discussion

The use of humanized murine Tg-AD models has significantly increased our understanding of the role of the pathological mechanisms that drive amyloidogenesis and inflammatory degeneration characteristic of the AD phenotype [6–10]. In the AD brain A β 42 peptides progressively accumulate and self-aggregate, evolving into higher order proinflammatory senile plaque deposits; in progressive neurodegenerative diseases of the retina such as age-related macular degeneration (AMD), A β 42 peptides accumulate with age and self-aggregate into highly insoluble pro-inflammatory lesions called drusen [27–32]. The inflammatory biomarker COX-2 (EC 1.14.99.1; MW~72 kDa) is an inducible, NF- κ B-regulated isotype of prostaglandin-endoperoxide synthase, responsible for the formation of several prostanoid-types of inflammatory mediators including prostaglandins prostacyclins, thromboxanes and leukotrienes in the brain and retina [20,29,30]. As the rate-limiting enzyme of the arachidonic acid cycle, COX-2 is significantly elevated in anatomical regions targeted in the AD brain where it in turn upregulates prostanoids that drive inflammatory neuropathology [29,30]. Elevations in CRP (P02741; also known as pentraxin 1; MW~25.1 kDa), a highly soluble serum protein and sensitive biomarker for systemic inflammatory disease, has recently been shown to promote A β 42 peptide production and amyloidogenesis [30,31]. The parallel and coordinated up-regulation of A β 40 and A β 42 peptides, COX-2 and CRP in aluminum-treated 5xFAD animals implicates an aluminum-orchestrated increase of

pro-inflammatory signaling in both the brain cortex and retina of 5xFAD mice [3–6]. Indeed multiple previous studies have shown that aluminum exerts significant pro-inflammatory effects in a variety of embryologically-related cell types of the mammalian CNS [4,10–17,25,32,33]. Interestingly, the common neuroectodermal origins of the mammalian cortex and retina may predispose these highly integrated, structurally similar, multi-layered cellular structures to AD-type dysfunction, including the involvement of shared pathogenic signaling pathways that drive amyloidogenesis, inflammatory neurodegeneration and the progressive deposition of aggregates of highly insoluble A β 42-peptides.

Over the last several years a picture has begun to emerge of the AD as an ‘evolving’, ‘advancing’, ‘spreading’ or ‘propagating’ amyloidogenic and pro-inflammatory, ultimately fatal, CNS dysfunction eventually manifesting as amore ‘globalized’ CNS disorder than previously appreciated. There is very recent evidence that AD involves not only the brain's limbic system, but primary visual cortical regions, the thalamus, and eventually the sensory retinal ganglia, especially in as AD progresses into its later stages [2–8]. Interestingly, the anatomical linkage from the brain's limbic system to the primary visual cortex, to the thalamus, and from the thalamus to the retinal ganglion of the posterior retina can involve as few as three neurons and their processes [6,27,28,31,32,34]. The current results further suggest that in Tg-AD models which are genetically predisposed to developing AD-type amyloidogenesis, that aluminum not only accelerates AD-type brain pathology but that other primary sensory organs such as the retina of the eye are also affected. The brain cortex and retina of the aging 5xFAD model may be useful in following the spread of aluminum-mediated pathology along the cerebral–thalamic–retinal axis, and slowing this progression is an attractive therapeutic strategy for the clinical management of AD even if a complete cure for AD cannot be found [5–8,32,34–36]. It will be certainly be interesting to investigate whether the pro-inflammatory molecular changes in the aluminum-fed 5xFAD mouse retina correlate to the progressive visual field deficits as is observed in late-stage AD patients, and to further evaluate whether advanced retinal imaging techniques might have any value in predicting who may be at risk for developing AD [6,34–36].

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Fig. 1.

Increased COX-2 and CRP in the retina of aging control- and aluminum-fed 5xFAD Tg-AD mice — whole retina of control 0 month (newborn pups), 1.5, 3 and 5 month old 5xFAD Tg-AD mice (control-fed) or those receiving aluminum-supplemented diets (aluminum-fed) were analyzed for the inflammatory biomarkers COX-2 and CRP; basal levels of COX-2 and CRP in newborn 5xFAD Tg-AD animals were set to 1.0 (dashed horizontal line for ease of comparison); 3 and 5 month old aluminum-fed 5xFAD mice showed 6.2- and 7.5-fold increases in COX-2 levels over controls; similarly 3 and 5 month old aluminum-fed 5xFAD mice showed 13.5- and 14.2-fold increases in CRP levels over controls; the increases in COX-2 and CRP in aluminum-fed animals were highly significant especially after the 1.5month time point when A β 42 peptides begin to rapidly accumulate [1–4,28,31]; previous reports from our lab have shown elevated beta amyloid precursor protein (β APP) and A β 42 peptide levels in the thalamus and primary visual cortex (Brodmann area A17) of advanced AD patients [3,4,6]; N=3 to 5 animals per time point; * p < 0.05; ** p < 0.001 (ANOVA).

Aβ40 and Aβ42 peptide, COX-2 and CRP levels in aging 5xFAD Tg-AD brain and retina.

Table 1

Strain/tissue	AI	N	Aβ40 peptide (pmol/g protein)	Aβ42 peptide (pmol/g protein)	Aβ40 + Aβ42 (pmol/g protein)	COX-2	CRP
Control brain	-	5	2.23 +/- 1.1	14.15 +/- 2.01	16.28	1	1
Control retina	-	5	1.31 +/- 0.8	2.45 +/- 4.51	3.5	1	1
5xFAD brain	-	4	861.1 +/- 451*	1412 +/- 217* (100x)	2273.1* (140x)	4.2*	7.1*
5xFAD retina	-	4	5.6 +/- 1.5*	265.2 +/- 102* (128x)	270.8* (91x)	3.1*	5.2*
5xFAD brain	+	4	910.3 +/- 378*	1965 +/- 1310.5* (139x)	2875* (177x)	7.4*	13.7*
5xFAD retina	+	4	13.8 +/- 2.4	387.1 +/- 123* (158x)	401* (115x)	6.1*	15.5*

Statistical analysis of Aβ40 and Aβ42 peptide load and COX-2 and CRP in 3month old 5xFAD brain and retina; numbers for Aβ peptides (expressed as pmol/g of protein) are the mean plus one standard deviation from that mean; only the means of COX-2 and CRP levels are shown (rightmost columns); control animals were from C57BL/6SJL founder strains; all control brain and retina COX-2 and CRP levels were set to '1' for ease of comparison; the combined Aβ40+Aβ42 peptide abundance is an arithmetic sum of individual Aβ40 plus Aβ42 peptide mean abundances; Aβ40+Aβ42 peptides in 3 month old 5xFAD mice were found to be 140 times the abundance over control brain and 91 times the abundance in (whole) retina over controls, 177 times the abundance in aluminum-fed 5xFAD brain cortex over control and 115 times the abundance in aluminum-fed 5xFAD (whole) retina over control; Aβ42 increases in the aluminum-fed 5xFAD retina over age-matched controls are even more pronounced than Aβ40 changes; notably, Aβ42 is considerably more fibrillogenic and neurotoxic than the shorter Aβ40 peptide [4,16,27]; Aβ42 peptide increases were 128x over control in 5xFAD mouse retina receiving normal diets, and 158x over control in 5xFAD mice receiving aluminum-supplemented diets; increases of Aβ40+Aβ42 peptides (and especially Aβ42 peptides) in 5xFAD (whole) retina are highly significant, suggesting that Aβ40+Aβ42 peptide accumulation (and in particular Aβ42 peptide increases) in the retina is a progressive and age-related extension of amyloidogenic pathology in the brain; see text for discussion of COX-2 and CRP levels; AI: - or +; * indicates absence or presence of aluminum in the diet, respectively; N = number of animals used in each group; significance over respective controls is indicated by single asterisks as *p < 0.01 (ANOVA); see also Refs. [3-6,10-17].