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Blocking the Apolipoprotein E/Amyloid- β Interaction Reduces Fibrillar Vascular Amyloid Deposition and Cerebral Microhemorrhages in TgSwDI Mice

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Abstract

The accumulation of amyloid- β (A β) peptides as toxic oligomers, amyloid plaques, and cerebral amyloid angiopathy (CAA) is critical in the pathogenesis of Alzheimer's disease (AD). The binding of A β peptides to apolipoprotein E (ApoE) plays an important role in modulation of amyloid deposition and clearance. We have shown that blocking the A β /ApoE interaction with A β _{12-28P}, a nontoxic blood-brain-barrier permeable and non-fibrillogenic synthetic peptide, constitutes a novel therapeutic approach for AD by reducing A β parenchymal deposition. In the present study, we investigate this therapeutic effect on CAA in the transgenic (Tg) AD mice model (TgSwDI), which expresses Swedish (K670N/M671L), Dutch (E693Q)/Iowa (D694N) A β PP mutations. These mice develop abundant CAA beginning at the age of 6 months. Behavioral results show that A β _{12-28P} treated TgSwDI AD mice performed the same as wild-type mice, whereas vehicle treated TgSwDI were impaired in spatial memory. Furthermore, this treatment resulted in a significant reduction of total amyloid burden, especially the fibrillar vascular amyloid burden, which importantly was accompanied by a reduction in microhemorrhages and neuroinflammation. Measurement of A β levels in the brain homogenate revealed a significant decrease in both the total amount of A β and A β oligomer levels in A β _{12-28P} treated TgSwDI mice. These findings suggest that blocking the A β /ApoE interaction is a highly effective therapeutic approach for vascular amyloid deposition, in contrast to some other therapeutic approaches.

Keywords

Alzheimer's disease; amyloid- β ; apolipoprotein E; cerebral amyloid angiopathy; microhemorrhages; microglia; neuroinflammation

INTRODUCTION

The extracellular accumulations of amyloid- β (A β) peptides as plaques and cerebral amyloid angiopathy (CAA), as well as intracellular neurofibrillary tangles, are pathological hallmarks of Alzheimer's disease (AD). Extensive evidence supports the amyloid cascade hypothesis in which A β accumulation and aggregation in the brain are central events in the pathogenesis of AD [1, 2]. A β peptides are derived through sequential enzymatic cleavage of the amyloid- β protein precursor (A β PP) by β - and γ -secretases. Either increased production of A β peptides or their inadequate clearance by cellular uptake or transport across the blood-brain-barrier (BBB), leads to the accumulation of A β peptide in the form of plaques in brain parenchyma and in walls of cerebral vessels as CAA.

CAA occurs in about 98% of AD patients, with approximately 75% of these cases rated as severe CAA [3–5]. In addition, CAA is present in about 30% of non-demented elderly [3]. Clinical studies have shown a strong correlation between cognitive impairment and the presence of CAA [6–8]. Furthermore, CAA is associated with focal ischemia and cerebral hemorrhage due to weakening of vascular walls by amyloid deposits and focal inflammation. Specific point mutations within the A β peptide have been identified, including Dutch-type (E22Q) and Iowa-type (D23N), that cause familial forms of CAA [9, 10]. A transgenic mouse model has been generated expressing human Swedish, Dutch, and Iowa triple A β PP mutations (TgSwDI) in brain. TgSwDI mice show early-onset cerebral microvascular amyloidosis in form of fibrillar vascular A β deposits starting at the age of 6 months [11]. Moreover, CAA in TgSwDI mice is linked with a robust neuroinflammation and deficits in spatial memory. TgSwDI model mice provide a critical tool to understand the effects of amyloid deposition in the vasculature and to study therapeutic interventions in CAA.

ApoE is a known genetic risk factor for both AD and CAA. Inheritance of the ApoE4 allele is the strongest genetic risk factor identified so far for late-onset AD. Carrying an ApoE4 allele increases the incidence and decreases the age of onset of AD pathology and CAA [12, 13]. A number of studies have shown that ApoE and A β bind *in vivo* and *in vitro*, with this binding affecting the conformation of A β peptides, highlighting the importance of this interaction for the modulation of aggregation and clearance of A β [14–20]. A β PP_{SW}E and PDA β PP mice bred onto an ApoE-null background results in complete absence of CAA and associated microhemorrhages [21]. Similarly, crossing TgSwDI mice onto an ApoE knock out (KO) background greatly reduces the fibrillar cerebral microvascular A β deposits with decreased neuroinflammation [22]. These observations demonstrate that ApoE is essential for vascular amyloid formation in transgenic mice. Therefore, pharmacological blockade of the ApoE/A β interaction may provide an alternative therapeutic strategy for CAA.

To block this binding, we synthesized a peptide, A β _{12-28p}, which is homologous to the binding site of ApoE on A β but substituted valine to proline at residue 18. A β _{12-28p} is a nontoxic, non-fibrillogenic and blood-brain-barrier (BBB) permeable peptide with a serum half-life of 62 ± 7 min (mean $\pm$ SEM). Our previous studies demonstrate that A β _{12-28p} reduces A β toxicity *in vitro* by preventing binding to ApoE, that it is able to cross the BBB and that it reduces amyloid deposition in two transgenic AD mice models (APP_{K670/M671L} and APP_{K670/M671L}PS1_{M146L} Tg mice) with primarily parenchymal amyloid deposition [23, 24]. In the present study, our aim is to assess the therapeutic effect of A β _{12-28p} on the extensive CAA deposition found in TgSwDI mice.

MATERIALS AND METHODS

Synthesis of peptides

The A β ₁₋₄₀ and A β _{12-28p} peptides were synthesized at the W.M. Keck Foundation Laboratory (Yale University, New Haven, CT). Details of synthesis and sequence verification were described previously [23]. Briefly, the A β _{12-28p} (VHHQKLPPFAEDVGSNK) peptide used for treatment was synthesized using D-amino acids, end protected by C-terminal amidation and N-terminal acetylation to extend the serum half-life. To make sure that A β _{12-28p} is non-toxic, its secondary structure was evaluated using circular dichroism as described previously [19].

Transgenic mice

The treatment was performed on transgenic mice expressing human Swedish K670N/M671L, Dutch E693Q, and Iowa D694N A β PP mutations (TgSwDI). These mice show early-onset and extensive fibrillar A β deposits in cerebral blood vessels, with mainly diffuse A β deposition in the brain parenchyma starting at the age of 3 month [11]. TgSwDI mice were obtained from the Jackson Laboratory (Bar Harbor, ME). Both TgSwDI mice and wild-type (WT) control mice were littermates on a pure C57BL/6 background. All mouse care and experimental procedures were compliant with guidelines of animal experimentation and were approved by the Institutional Animal Care and Use Committee at the New York University School of Medicine.

Treatment administration

TgSwDI mice were injected intraperitoneally (i.p.) with 1 mg of A β _{12-28p} diluted under sterile condition in 0.5 ml of normal saline three times per week for 6 months, beginning at the age of 3 months. Age-matched vehicle (saline alone) treated TgSwDI mice and WT mice were used as controls. Seven mice were included in each group. During the treatment, veterinary staff monitored animals for any signs of toxicity, such as changes in body weight, physical appearance, and altered behavior. Animals were killed a week after administration of the last dose of A β _{12-28p}. At the time of death, samples of the heart, lungs, liver, kidney, spleen, and skeletal muscles were collected, fixed, embedded in paraffin, and stained with hematoxylin/eosin and Congo red for detection of systemic amyloidosis or any other sign of toxicity. No toxicity was evident in the A β _{12-28p} treated group.

Behavior testing

Locomotor activity—Before cognitive testing, exploratory locomotor activity was measured to verify that any treatment effects observed in the cognitive task could not be explained by differences in motor abilities. A Hamilton-Kinder Smart-frame Photobeam System was used to record animal activity over a designated period of time [25]. Mice were placed in a circular open field chamber (70 × 70 cm) and allowed to explore the environment for 15 min. Horizontal movements of the animal were automatically recorded by a video camera mounted above the chamber. Results were reported as distance traveled, travel velocity (average and maximum) and mean resting time of the animal.

Radial arm maze—Spatial memory was tested using a radial arm maze, as described previously [24–26]. The apparatus consisted of eight radial 30-cm-long arms originating from the central space. A water well with 0.25 ml of 0.1% saccharine solution was placed at the end of each arm. Before testing, mice were deprived of water for 24 h and then their access to water was restricted to 2 h per day for the duration of testing. The task required an animal to enter all arms and drink the saccharine solution until the eight rewards had been consumed. After 4 days of adaptation, mice were subjected to testing for 10 consecutive

days. The number of errors (entry into previously visited arms) was recorded during each testing session. This test was performed during the last month of the experiment while animals were still receiving A β _{12-28p} or vehicle. The behavioral testing was performed by an individual blinded to the experimental condition.

Histological studies

Mice were anesthetized with sodium pentobarbital (150 mg/kg, i.p.) and perfused transaortically with 0.1 M PBS, pH 7.4. Brains were immediately removed as described previously [24, 26]. The right hemisphere was immersion-fixed in periodate-lysine-paraformaldehyde (PLP), whereas the left hemisphere was snap-frozen for measurement of A β oligomers and A β levels. After fixation, brains were placed in 2% DMSO/20% glycerol in PBS and stored until sectioned. Serial coronal brain sections (40 μ m) were cut and stained for immunohistochemical analysis with: (1) a mixture of anti-A β antibodies 6E10/4G8 (1 : 1000, Covance), (2) rabbit polyclonal antibody to collagen IV (1 : 400, Abcam), (3) Thioflavin-S, (4) anti-mouse apoE antibody M-293 (1 : 200, Santa Cruz), (5) glial fibrillary acidic protein (GFAP) antibody for the detection of astrocytes (1 : 1000, Dako), (6) anti-CD11 b antibody (1 : 600, Serotec) and (7) anti-CD45 antibody (1 : 1000, Serotec) for the detection of activated microglia. We choose to use two different anti-A β monoclonal antibodies with distinct epitopes for amyloid detection to ensure that in A β _{12-28p} treated mice reductions of amyloid detection could not be attributed to epitope masking. Details of the immunostaining techniques were reported previously [22, 24, 26, 27]. Primary antibodies were detected with horseradish peroxidase-conjugated or alkaline phosphatase-conjugated secondary antibodies and visualized with either 3, 3'-diaminobenzidine and nickel ammonium sulfate intensification or with the fast red substrate, respectively. Thioflavin-S staining for fibrillar amyloid was performed on mounted sections, as published previously [24]. Perl's iron staining was performed on another set of sections to detect cerebral microhemorrhages. Sections were stained in a solution containing 5% potassium ferrocyanide and 10% hydrochloric acid for 40 min.

Quantitative analysis of amyloid deposition

Immunohistochemistry of tissue sections were quantified with a Bioquant stereology image analysis system (R&M Biometrics) using a random unbiased hierarchical sampling scheme, as published previously [23, 26]. All measurements were performed by an individual blinded to group assignment. Total A β burden (defined as the percentage of test area occupied by A β) was quantified for cortex, hippocampus, and thalamus on coronal plane sections double-stained with 6E10/4G8 and collagen IV. The cortical area was measured as dorsomedial from the cingulate cortex and extended ventrolaterally to the rhinal fissure within the right hemisphere. Test areas (640 μ m $\times$ 480 μ m) were randomly selected by applying a grid (800 μ m $\times$ 800 μ m) over the traced contour. About 25 areas were analyzed per animal. Hippocampal and thalamic measurements (600 μ m $\times$ 600 μ m) were performed similarly to the cortical analysis [24, 26]. Total fibrillar A β burden was evaluated in sections stained with Thioflavin-S using methods described previously [24, 26]. Several studies have demonstrated that, in TgSwDI mice, fibrillar amyloid deposits are almost exclusively localized to cerebral microvasculature, with mainly diffuse parenchymal amyloid deposits [11, 22, 28]. The rare Thioflavin S positive deposits which were in the brain parenchyma were excluded manually; therefore, the fibrillar A β burden corresponded to the fibrillar vascular amyloid burden. For quantifying ApoE and A β colocalization, ApoE-positive deposits were evaluated on sections immunostained for ApoE and Thioflavin S stained for fibrillar A β in an analogous manner to that used to measure the A β burden. The amount of colocalization was expressed as a percentage of the fibrillar A β burden. Perl's staining was assessed by a semiquantitative analysis method as published previously [26]. Ten sections were analyzed per mouse and the average number of iron positive profiles per section was

calculated. Images of histological sections obtained from the microscope for quantitative studies were not subject to any postprocessing.

Quantitative analysis of activated microglia and reactive astrocytosis

The CD45 microglial burden (the percentage of area occupied by CD45 reactive microglia) was quantified in a manner analogous to that used to measure the total A β burden. The assessment of the CD11 b stained sections was based on a semiquantitative analysis of the extent of microgliosis (0, a few resting microglia; 1, a few ramified and/or phagocytic microglia; 2, moderate number of ramified/phagocytic microglia; 3, numerous ramified/phagocytic microglia), as we have described previously [26]. Reactive astrocytosis was rated on a scale of 0.5–3. The rating was based on a semiquantitative analysis of the extent of GFAP immunoreactivity (number of GFAP immunoreactive cells and complexity of astrocytic branching), as we have previously published [26, 27].

Sandwich ELISA for A β levels

Extraction of A β from brains was performed as described previously [25, 26]. Brains were weighed and homogenized (10% w/v) in tissue homogenization buffer (20 mM Tris base, pH 7.4, 250 mM sucrose, 1 mM EDTA, 1 mM EGTA) with 100 mM phenyl-methylsulphonyl fluoride and protease inhibitors [protease inhibitors cocktail (Complete, Roche Diagnostic) as well as pepstatin A] added immediately before homogenization. For extraction of soluble A β , brain homogenates were mixed with an equal volume of cold 0.4% diethylamine (DEA)/100 mM NaCl, and subsequently centrifuged at 100,000 $\times$ g for 1 h at 4°C, as previously reported [24, 29, 30]. Then, the supernatant was neutralized with 1/10 volume of 0.5 M Tris, pH 6.8. The samples were then aliquoted, flash-frozen on dry ice, and stored at –80°C until used for ELISA analysis. For extraction of the total A β , homogenates (200 μ l) were added to 440 μ l of cold formic acid (FA) and sonicated for 1 minute on ice. Subsequently, 400 μ l of this solution was spun at 100,000 $\times$ g for 1 h at 4°C. Then, 210 μ l of the resulting supernatant was diluted into 4 ml of FA neutralization solution (1 M Tris base, 0.5 M Na₂HPO₄, 0.05% NaN₃), aliquoted, flash-frozen on dry ice, and stored at –80°C until used for A β measurement.

The total A β level and soluble A β fraction level were measured by sandwich ELISA which uses 6E10 monoclonal antibody as a capture antibody and two rabbit polyclonal antibodies specific for A β ₄₀ (R162) and A β ₄₂ (R165), as described previously [26, 31]. The assay was performed by a person (PM) who was blinded to treatment group assignment. The levels of A β species are presented as μ g of A β per g of wet brain, taking into account the dilution during brain homogenization and extraction procedures.

Western blot analysis of A β oligomers

Samples of A β homogenate were centrifuged at 100,000 $\times$ g for 1 h at 4°C. The supernatants were collected and the protein concentrations were determined using the bicinchoninic acid assay (BCA; Pierce, Rockford, IL). Thirty-five μ g of each sample, mixed with an equal volume of Tricine sample buffer, was loaded per lane and subjected to overnight electrophoresis on 12.5% SDS-polyacrylamide Tris-tricine gels under non-reducing conditions. A β oligomers were detected by using oligomer-specific A11 polyclonal antibody (Biosource, Camarillo, CA) [24]. The specificity of A11 staining was confirmed by probing the membranes with anti-A β antibodies 6E10/4G8. Densitometric analysis of A β oligomer bands was performed with NIH Image J software (version 1.34).

ELISA for anti-A β antibodies

Sera of A β _{12-28P} treated mice were tested for the presence of antibodies against A β _{1-40/42} and A β _{12-28P}. Sera in dilutions of 1 : 200 and 1 : 500 were applied to 96-well microtiter plates coated with synthetic A β ₁₋₄₀ or A β _{12-28P} as described previously [24, 26]. The plates were incubated with goat anti-mouse IgG antibodies conjugated with horseradish peroxidase, then developed with tetramethyl benzidine substrate (TMB; Pierce). The color reaction was stopped with 2 M sulfuric acid, and the absorbance was read at 450 nm wavelength.

Statistical analysis

Data from the radial arm maze test were analyzed by repeated-measures ANOVA followed by Neuman–Keuls *post hoc* tests. Data from locomotor activity, and GFAP astrogliosis were analyzed using one-way ANOVA. Differences between groups in total amyloid burden, fibrillar amyloid burden, levels of extracted A β , levels of A β oligomers, brain microhemorrhages, co-localization of ApoE and A β , CD11 b and CD45 for activated microglia were analyzed using unpaired two-tailed *t* tests. All statistical analysis was performed using Prism 5.0 (GraphPad, San Diego, CA).

RESULTS

Behavioral studies

The A β _{12-28P} treated mice, vehicle- treated Tg mice and WT controls were assessed on both cognitive and locomotor tests. No statistical differences were observed between groups in any of the locomotor parameters measured (Fig. 1). TgSwDI mice showed no changes in exploratory movements compared with WT mice, which is consistent with other studies [28]. Therefore, the performance on cognitive tests was not influenced by motor abnormalities.

TgSwDI mice treated with A β _{12-28P} navigated the radial arm maze much better than the vehicle-treated transgenic mice [Fig. 2; repeated-measures ANOVA: treatment effect, $p < 0.0001$; days effect, $p = 0.0084$; interaction (treatment vs. days), $p = 0.99$]. The A β _{12-28P} treated TgSwDI mice performed comparably to WT mice, whereas vehicle-treated Tg mice made significantly more errors than WT mice (Newman–Keuls *post hoc* test, A β _{12-28P} vs. vehicle, $p < 0.001$; A β _{12-28P} vs. WT, $p > 0.05$; vehicle vs. WT, $p < 0.001$). A prior study has characterized that TgSwDI mice exhibit spatial memory deficits as early as 3 months of age [28]. Therefore, our result suggests that the treatment with A β _{12-28P} prevented memory deficits in TgSwDI mice.

Quantification of A β burden

Total amyloid burden in the regions of cortex, hippocampus, and thalamus were quantified by stereological techniques, using random unbiased sampling on the immunostained serial sections (Fig. 3). Peripheral administration of A β _{12-28P} led to 70% (two-tailed *t* test, $p < 0.001$) reduction in total cortical amyloid burden, 77% ($p < 0.0001$) reduction in hippocampal amyloid burden, and 77% ($p < 0.0001$) reduction in thalamus, compared with age-matched control Tg mice that received vehicle only (Fig. 4).

Thioflavin-S staining reveals only the fibrillar amyloid deposition and not diffuse preamyloid lesions [32–34], which in this model corresponds almost exclusively to the vascular amyloid burden [11, 22, 28]. Quantitative assessment of Thioflavin-S staining revealed 90% (two-tailed *t* test, $p < 0.0001$) reduction in the total cortical fibrillar vascular amyloid burden (Fig. 5). 76.7% and 83.1% reductions of the total fibrillar vascular amyloid burden were observed in the hippocampus and thalamus, respectively (two-tailed *t* test,

hippocampus, $p = 0.0004$; thalamus, $p < 0.0001$). These results are consistent with the findings of a nearly complete elimination of fibrillar amyloid deposits in apoE knockout TgSwDI mice [22].

Brain microhemorrhages were detected in brain sections of TgSwDI mice stained with Perl's staining (Fig. 6). After treatment with A β _{12-28p}, there was a significant decrease in the extent of cerebral microhemorrhages in TgSwDI mice (two-tailed t test, $p = 0.0016$).

Assessment of A β levels and A β oligomers in the brain

ELISA measurements revealed a statistically significant decrease in the levels of total (FA extracted) A β ₄₀ and A β ₄₂ by 63.3% (two-tailed t test, $p = 0.0078$) and 63.8% ($p = 0.0086$), respectively, after the treatment with A β _{12-28p} (Fig. 7A). The levels of soluble (DEA extracted) A β ₄₀ and A β ₄₂ fractions were significantly reduced by 42% (two-tailed t test, $p = 0.0198$) and 50% ($p = 0.0116$), respectively, in A β _{12-28p} treated mice (Fig. 7B).

Soluble oligomeric A β ligands may account for memory loss and AD neuropathology, thus presenting a significant therapeutic target. The levels of pathogenic A β oligomers in the brain homogenates were assessed by Western blot using the A11 oligomer-specific antibody (Fig. 8). A β _{12-28p} treatment led to a significant decrease in the levels A11 reactive oligomers (~45 kDa) band (two-tailed t test, $p < 0.001$).

Decreased neuroinflammatory response after treatment

Abnormal activation of microglia and astrocytes is observed in the brains of AD patients and transgenic AD mouse models [26, 35, 36]. Previous studies demonstrate that the deposition of cerebral microvascular amyloid was accompanied by large increases in the numbers of neuroinflammatory reactive astrocytes and activated microglia in TgSwDI mice [22, 28]. We evaluated the treatment effect of A β _{12-28p} on inflammation by immunohistochemical staining.

To quantitatively examine the extent of gliosis, we assessed the activation of microglia using CD45 (Fig. 9) and CD11 b (Fig. 10) as markers. Wild-type mice showed no activated microglia in any of the brain regions (data not show). On the other hand, TgSwDI mice presented numerous activated microglia in different brain regions, strongly associated with the fibrillar amyloid burden (Figs 9 and 10). Larger numbers of activated microglia were found in the brain regions with the highest amounts of fibrillar amyloid deposition, such as hippocampus and thalamus (Fig. 5). Stereological analysis of CD45-positive microglia indicated a 72.4% reduction in the cortex, a 73.8% reduction in the hippocampus, and a 68% reduction in the thalamus of A β _{12-28p} treated TgSwDI mice compared with vehicle-treated Tg mice (Fig. 9G; two-tailed t test, cortex, $p = 0.0017$; hippocampus, $p < 0.001$; thalamus, $p = 0.0015$). The assessment of microglial marker CD11 b was based on semiquantitative analysis of the extent of microgliosis. There was a significant reduction in the CD11b-positive activated microglial burden in TgSwDI mice treated with A β _{12-28p}, compared with vehicle-treated Tg mice (Fig. 10G; two-tailed t test, cortex, $p = 0.0036$; hippocampus, $p = 0.0442$; thalamus, $p = 0.0023$).

In addition, brain sections were stained with GFAP to quantify the extent of astrogliosis (Fig. 11). Modest astrocyte staining was observed in cortex of the A β _{12-28p} treated Tg mice and wild-type mice. Vehicle-treated TgSwDI mice exhibited numerous strongly GFAP-positive reactive astrocytes. Semiquantitative analysis of astrogliosis in the cortex revealed fewer activated astrocytes in A β _{12-28p} treated mice compared with vehicle treated mice (one-way ANOVA, $p < 0.0001$; *post hoc* A β _{12-28p} vs. vehicle, $p < 0.0001$; WT vs. vehicle, $p < 0.0001$; A β _{12-28p} vs. WT, $p > 0.05$). Reactive astrogliosis was also decreased in the hippocampus and thalamus of A β _{12-28p} treated Tg mice (hippocampus: one-way ANOVA, p

< 0.0001 ; *post hoc* $A\beta_{12-28P}$ vs. vehicle, $p < 0.001$; WT vs. vehicle, $p < 0.001$, $A\beta_{12-28P}$ vs. WT, $p < 0.05$; thalamus: one-way ANOVA, $p < 0.0001$; *post hoc* $A\beta_{12-28P}$ vs. vehicle, $p < 0.0001$; WT vs. vehicle, $p < 0.0001$; $A\beta_{12-28P}$ vs. WT, $p > 0.05$).

Quantification of ApoE associated A β deposits

To determine if the treatment effect was associated with a decreased interaction of ApoE/A β , we quantified colocalization of ApoE immunoreactivity and Thioflavin stained amyloid deposits (Fig. 12). A mean of 76.7% (± 9.6 SEM) of ApoE in thalamus was associated with fibrillar A β in vehicle-treated Tg mice, whereas only 26.6% (± 6.0) of the ApoE in $A\beta_{12-28P}$ treated mice was associated with A β (two-tailed t test, $*p = 0.004$). There were also significant reductions in the ApoE associated A β deposits in the hippocampus and cortex of TgSwDI mice treated with $A\beta_{12-28P}$ compared with vehicle-treated Tg mice (hippocampus $65\% \pm 9$ in vehicle treated versus $27\% \pm 6.2$ in $A\beta_{12-28P}$ treated, $**p = 0.007$; cortex $62\% \pm 10$ in vehicle treated versus $36\% \pm 6.4$ in $A\beta_{12-28P}$ treated, $*p = 0.0175$) (Fig. 12).

Anti-A β antibody levels

An immune response against $A\beta_{12-28P}$ and $A\beta_{40/42}$ was monitored to assure that the treatment effect was not associated with a vaccination effect. No anti-A β antibodies were detected in the sera of TgSwDI mice treated with either $A\beta_{12-28P}$ or vehicle. This indicates that the effect of $A\beta_{12-28P}$ on amyloid burden cannot be attributed to a humoral response against A β .

DISCUSSION

CAA is a critical part of AD pathology [37]. The vast majority of AD patients have some degree of CAA at autopsy, with about 75% having “severe” CAA [3, 4]. Furthermore, CAA is present in about 30% of cognitively normal elderly, control populations [3, 38]. The population based Honolulu Asia Aging Study has shown the presence of CAA to be an independent risk factor for the presence of dementia [6, 8]. There are numerous Tg models of parenchymal amyloid deposition which also have some degree of vascular amyloid deposition with a variable age of onset [39]. However, the Tg model with the most extensive vascular amyloid in association with lower levels of parenchymal amyloid is the TgSwDI mouse which incorporates 3 A β PP mutations: Swedish, Dutch E693Q and Iowa D694N, that we used in this study [11, 28, 40]. These Dutch and Iowa mutations are associated with hereditary cerebral hemorrhage with amyloidosis (HCHWA) [9, 10]. The parenchymal A β deposits in these mice largely do not stain with Congo red and represents non-fibrillar, diffuse amyloid similar to the neuropathology of HCHWA-Dutch [41]. A caveat with the TgSwDI mice is that the vascular amyloid deposition is mainly in capillaries, as shown in previous studies of this model [11, 28, 40, 42], contrasting with AD where CAA is more frequent in arterioles and there is less capillary involvement. Interestingly in human autopsy tissue it is the capillary CAA level which correlates best with the presence of other AD related pathology [43] as well as with the presence of ApoE4 allele [44], indicating the biological importance of this subtype of CAA and the relevance of this Tg model of CAA.

In the current study we sought to determine if treatment of TgSwDI mice with $A\beta_{12-28P}$ would lead to a reduction of CAA in the absence of microhemorrhages or increased inflammation. We had previously tested $A\beta_{12-28P}$ in Tg mice carrying the K670/M671L A β PP mutation ($A\beta$ PP_{SWE} or Tg2576 mice) and in double Tg mice also carrying the presenilin 1 M146L mutation ($A\beta$ PP_{SWE/PS1}) from the ages of 12 to 18 months and 2 to 7 months, respectively [24]. We document that $A\beta_{12-28P}$, by blocking the interaction between apoE and A β , was able to significantly reduce the parenchymal amyloid burden. The CAA burden was also significantly reduced in the $A\beta$ PP_{SWE} mice; however, these mice had

mainly parenchymal amyloid deposition with the CAA being minimal [24]. The analysis of CAA in the 7 month old A β PP_{SWE}/PS1 was confounded by the very low and variable CAA burden in these mice at this age, and we were unable to document any significant CAA reduction. In the current study we were able to show a 90%, 76.7% and an 83.1% reduction in the cortical, hippocampal and thalamic fibrillar vascular amyloid burdens, respectively. This was associated with a behavioral rescue in the treated mice on radial arm maze testing ($p < 0.0001$ treatment effect), with there being no significant difference between A β _{12-28p} treated TgSwDI mice versus WT mice on *post-hoc* testing. This cognitive testing was not confounded by any locomotor differences between the mouse groups. This cognitive improvement was coupled with reductions of both the soluble and insoluble A β peptide levels. This is striking in that many prior amyloid reducing strategies document reductions of insoluble A β but not of soluble A β [24, 25, 46]. Significantly there was also a reduction of A β oligomers. A potential risk associated with approaches that target A β deposition involves increasing the pool of soluble A β which may in turn favor formation of the toxic oligomer species. We demonstrate that at least in this model, this is not the case. It is also a concern that targeting A β deposition may lead to increased brain inflammation with resultant neuronal dysfunction/death. Our studies document that CD45, CD11b, and GFAP immunoreactivity is markedly reduced in the A β _{12-28p} treated Tg mice.

We document that the likely mechanism of action of A β _{12-28p} is by blocking the interaction of ApoE and A β , in our quantitation of ApoE immunoreactivity co-localizing with Thioflavin positive amyloid deposits. Significant reductions of ApoE co-localization with amyloid deposits were noted in the cortex, hippocampus, and thalamus of A β _{12-28p} treated mice. A non-mutually exclusive, alternative mechanism of action for A β _{12-28p} is to inhibit A β peptide fibrillogenesis. However, in our past studies we have shown that over a wide range of concentrations A β _{12-28p} does not significantly inhibit A β ₁₋₄₀ or A β ₁₋₄₂ fibrillogenesis [23, 24, 46].

It is important that our treatment with A β _{12-28p} was able to markedly reduce CAA without cerebral microhemorrhage. Of the numerous A β deposition targeting approaches for the treatment of AD, among the most advanced is immunomodulation [45]. This approach is currently undergoing numerous human clinical trials from Phase I to Phase III [47]. A particular problem with this approach has been the lack of effectiveness of CAA clearance and the production of microhemorrhages. The mechanism of this hemorrhage is thought to be related to CAA that causes degeneration of smooth muscle cells and weakening of the blood vessel wall. A number of reports have shown an increase in microhemorrhages in several AD mouse models following passive immunization with different monoclonal antibodies with high affinity for A β plaques and CAA [48–52]. Microhemorrhages following active immunization in animal models have also been reported in several studies [48, 53–55]. In particular in the TgSwDI/NOS2 KO mouse model with the most extensive vascular amyloid, vaccination with A β ₁₋₄₂, while reducing the amyloid burden in association with behavioral benefits, led to a marked increase in micro-hemorrhages [55]. While this increase in microhemorrhages does not appear to be symptomatic in the mouse models, this would be much less likely in humans. Strategically placed microhemorrhages in patients have been shown to correlate with cognitive deficits [56, 57]. Furthermore, in the limited human autopsy data from patients who received active immunization there is evidence for increased CAA and microhemorrhages [58]. Hence this would suggest that treatment approaches such as A β _{12-28p} might be useful in addition to, or instead of immunomodulatory approaches to avoid these complications. A critical need exists for more effective forms of therapy for AD, in particular therapies that also target CAA and tau related pathology. Our data suggests that approaches which block the interaction between ApoE and A β with compounds such as A β _{12-28p} can be useful to specifically ameliorate

CAA related pathology without associated complications such as increased microhemorrhages.

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Fig. 1.

Shows an analysis of the locomotor activity of experimental mouse groups at 9 months of age. A–D) Both Tg groups and WT mice did not differ significantly in distance traveled (A), maximum speed (B), average speed (C) and rest time (D). The error bars show the SEM. (Colours are visible in the online version of the article; <http://dx.doi.org/10.3233/JAD-2011-101401>)

Fig. 2.

Shows the results of radial arm maze testing. TgSwDI mice treated with A β_{12-28P} navigated the radial arm maze with fewer errors than vehicle treated Tg mice, and performed as well as age-matched WT mice [treatment effect, $p < 0.0001$; days effect, $p = 0.0084$; interaction (treatment vs. days), $p = 0.99$. Newman-Keuls *post hoc* test, A β_{12-28P} vs. vehicle, $p < 0.001$; A β_{12-28P} vs. WT, $p > 0.05$; vehicle vs. WT, $p < 0.001$]. (Colours are visible in the online version of the article; <http://dx.doi.org/10.3233/JAD-2011-101401>)

Fig. 3. Shows double immunolabeling of A β (black) and collagen IV (red) in blood vessels at 9 months of age. Treatment with A β _{12-28P} decreased total amyloid burden in TgSwDI mice. Vehicle-treated Tg mice showed greater A β deposition in neocortex (A, B), hippocampus (C) and thalamus (D), compared with A β _{12-28P} treated Tg mice (E–H). Scale bars, 100 μ m. The scale bar in E corresponds to cortical image A and E; the scale bar in F corresponds to cortical image B and F; the scale bar in G corresponds to hippocampal image C and G; the scale bar in H corresponds to thalamic image D and H. (Colours are visible in the online version of the article; <http://dx.doi.org/10.3233/JAD-2011-101401>)

Fig. 4.

Quantitative image analysis of total amyloid deposition (amyloid burden) showed a significant reduction in TgSwDI mice treated with Aβ_{12-28P} compared with vehicle-treated Tg mice. There was a 70% reduction in cortex (** $p < 0.001$), a 77% reduction in hippocampus (*** $p < 0.0001$) and a 77% reduction in thalamus (*** $p < 0.0001$). (Colours are visible in the online version of the article; <http://dx.doi.org/10.3233/JAD-2011-101401>)

Fig. 5.

Shows a quantification of the fibrillar vascular amyloid burden. Thioflavin-S staining revealed overall significant differences in fibrillar vascular amyloid deposition between Aβ_{12-28P} treated TgSwDI mice (B, D, F) compared to vehicle treated Tg mice (A, C, E). G) Stereological analysis showed a 90% reduction in cortex ($***p < 0.0001$), a 76.7% in hippocampus ($**p = 0.0004$) and an 83.1% reduction in thalamus ($***p < 0.0001$). (Colours are visible in the online version of the article; <http://dx.doi.org/10.3233/JAD-2011-101401>)

Fig. 6. Shows quantitation of microhemorrhages assessed by the Perl's stain for ferric iron. Brain sections were stained with Perls' stain for ferric iron (blue) both in vehicle-treated (A) and in Aβ_{12-28P} treated (B) TgSwDI mice. The insert in A shows a higher power of cerebral microhemorrhages. The iron positive profiles per section decreased in Aβ_{12-28P} treated mice compared to vehicle-treated Tg mice as seen in C (two-tailed *t* test, ***p* = 0.0016). Scale bar = 100 μm. (Colours are visible in the online version of the article; <http://dx.doi.org/10.3233/JAD-2011-101401>)

Fig. 7.

Shows the quantitation of Aβ_{40/42} peptides in both the brain soluble and insoluble fractions. Treatment with Aβ_{12-28P} significantly decreased total (A) and soluble (B) Aβ levels in TgSwDI mice. A) The total Aβ levels of both Aβ₄₀ and Aβ₄₂ were reduced in Aβ_{12-28P} treated Tg mice compared with vehicle-treated Tg mice (Aβ₄₀, 63.3% reduction, ***p* = 0.0078; Aβ₄₂, 63.8% reduction, ***p* = 0.0086). B) The level of soluble Aβ₄₀ and Aβ₄₂ extracted with DEA revealed a significant difference between Aβ_{12-28P} treated and vehicle-treated Tg mice (Aβ₄₀, 42% reduction, **p* = 0.0198; Aβ₄₂, 50% reduction, **p* = 0.0116). (Colours are visible in the online version of the article; <http://dx.doi.org/10.3233/JAD-2011-101401>)

Fig. 8. Shows Western blot detection and densitometric analysis of an A11 oligomer specific band. A) Western blot of brain homogenates (35 μ g loaded in each lane) stained with A11 oligomer-specific antibody (on the left); the A11 immunoreactive band (~45 kDa) was also immunoreactive with anti-A β 6E10/4G8 antibodies (on the right). B) Densitometric analysis of an oligomer-specific band shows that the level of A11 reactive oligomers was reduced in A β_{12-28P} treated Tg mice compared with vehicle-treated Tg mice (two-tailed t test, $**p < 0.001$). (Colours are visible in the online version of the article; <http://dx.doi.org/10.3233/JAD-2011-101401>)

Fig. 9.

Shows CD45 immunoreactivity in Aβ_{12-28P} treated versus vehicle treated TgSwDI mice in neocortex (A, B), hippocampus (C, D) and thalamus (E, F). CD45 staining showed an overall reduction in microglial activity in Aβ_{12-28P} Tg treated mice. Quantitative stereological analysis revealed a 72.4% reduction in the cortex, a 73.8% reduction in the hippocampus, and a 68% reduction in the thalamus of Aβ_{12-28P} treated mice compared with vehicle treated mice (G; two-tailed *t* tests, cortex ***p* = 0.0017; hippocampus ***p* = 0.001; thalamus ***p* = 0.0015). The scale bars in B, D and F are 100 μm. Scale bars in B, D and F correspond to cortical, hippocampal and thalamic images, respectively. The insert shows a higher power image of activated microglia cells. The scale bar in the insert is 50 μm. (Colours are visible in the online version of the article; <http://dx.doi.org/10.3233/JAD-2011-101401>)

Fig. 10.

Shows CD11b immunoreactivity in Aβ_{12-28P} treated versus vehicle treated TgSwDI mice. Treatment with Aβ_{12-28P} reduced overall CD11b immunoreactivity in TgSwDI mice. Immunostaining with CD11b showed significant reductions in cortical (B), hippocampal (D) and thalamic (F) microgliosis in Aβ_{12-28P} treated compared with vehicle-treated (A, C, E) TgSwDI mice. Scale bars, 100 μm. (G) Shows the quantification of the CD11b immunoreactivity on a scale 0 to 3 (two-tailed *t* tests, cortex ***p* = 0.0036; hippocampus **p* = 0.0442; thalamus ***p* = 0.0023). (Colours are visible in the online version of the article; <http://dx.doi.org/10.3233/JAD-2011-101401>)

Fig. 11. Shows GFAP immunoreactivity in A β_{12-28p} treated versus vehicle treated TgSwDI mice. Treatment with A β_{12-28p} reduced cortical reactive astrogliosis in TgSwDI mice. GFAP staining (A–I) followed by semiquantitative analysis (J) revealed fewer activated astrocytes in A β_{12-28p} treated mice compared with vehicle-treated TgSwDI mice (one-way ANOVA $p < 0.0001$; *post hoc* tests: *** $p < 0.0001$, ** $p < 0.001$, * $p < 0.05$, ns: not significant). Scale bars, 100 μ m. (Colours are visible in the online version of the article; <http://dx.doi.org/10.3233/JAD-2011-101401>)

Fig. 12.

Shows colocalization of ApoE and fibrillar amyloid deposits in Aβ_{12-28P} treated versus vehicle treated TgSwDI mice. Brain sections from nine months old mice were labeled for fibrillar amyloid using thioflavin-S (green) and ApoE (red). Black arrows point to areas of co-localized ApoE and Aβ. The white arrows point to areas of thioflavin S staining, corresponding mainly to vascular amyloid, where there is no co-localized ApoE immunoreactivity. Quantitative analysis (G) showed ApoE and Aβ colocalization decreased in Aβ_{12-28P} treated (B, D, F) compared with vehicle-treated (A, C, E) TgSwDI mice (cortex, **p* = 0.0175; hippocampus, ***p* = 0.007; thalamus, ***p* = 0.004). Scale bars, 50 μm.