Inquiro, the UAB undergraduate science research journal

Volume 2013 | Number 7 Article 20

2013

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Abstract

Alzheimer's disease (AD) pathology is characterized by the deposition of a protein called amyloid β (Aβ) in plaques and blood vessel walls. It is thought that AD is not caused by Aβ deposits, but instead by the oligomeric form of Aβ. This study aimed to investigate the efficacy of two peptides, D3 and D3D3, in reducing these oligomers and the pathology they cause and in improving cognition. The D3 peptide is a D-enantiomer obtained from a mirror image phage display selection against small oligomeric forms of $A\beta_{42}$. D3D3 is a dimer of D3 connected by a peptide bond. D3 had previously been shown to reduce oligomers and improve pathology and cognition in AD transgenic (APP/PS1) mice. We hypothesized that the D3D3 peptide would bind more strongly than D3 to Aβ oligomers and therefore be more effective as a treatment. Because Aβ oligomers have been shown to start causing damage to the brain years prior to the onset of symptoms, we chose to test the peptides in young mice (4-6 months). Behavioral and cognitive analysis included Morris water maze, open field, and zero maze. Histological analysis involved staining for Aβ and measuring levels of Aβin the dentate gyrus and CA1 region of the hippocampus. Significant reduction in plaque accumulation was observed in the dentate gyrus and CA1 region with both peptides, but D3D3 was not superior to D3. Similarly, both experimental groups displayed significant improvement in cognitive functioning, but with no differences between D3D3 and D3.

Keywords: Alzheimer's disease, amyloid, limbic system, mouse, plaques, treatment

Introduction

Alzheimer's disease (AD) is a progressive neurodegenerative disease that affects millions of people worldwide. There are currently no curative treatments for this disease, and thus developing novel treatments is imperative. One of the key features of the disease is the accumulation of amyloid β (Aβ), a proteolytic fragment of amyloid precursor protein (APP), in plaques. These amyloid β deposits begin accumulating many years before the onset of cognitive symptoms. It was originally thought that Aβ plaques were the cause of the dementia; however, recent findings suggest that Aβ oligomers are the real cause of the synaptic damage that leads to disconnections within the brain.

Aβ monomers are released from the cell membrane and bind to each other, leading to the formation of Aβ fibrils that are deposited in plaques. Smaller forms of these aggregates, referred to as oligomers, also form. Evidence suggests that these Aβ oligomers are implicated in the degeneration of neural connections, i.e. synapses, leading to cognitive deficits. Thus, these Aβ oligomers were the target of this study. We hypothesized that long-term treatment with small peptides that bind to $A\beta_{42}$ (the most toxic form of Aβ) would result in changes in amyloid deposition in our transgenic (Tg) AD model mice. Our lab has previously tested a small D-enantiomeric peptide (D3) that was shown to reduce the accumulation of Aβ oligomers and improve cognition in AD transgenic (APP/PS1) mice.[1] This D3 peptide targets Aβ oligomers and was obtained from a mirror image phage display selection against small oligomeric forms of Aβ42.[2] Following this study, a second D-enantiomeric peptide (D3D3) was created with the intention of improving the effects of the first. A comparison of the two peptides was designed in order to test the efficacy of the new D3D3 peptide.

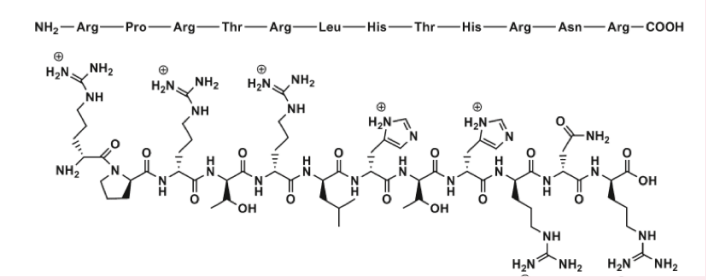

Figure 1. Amino acid sequence and chemical structure of the D3 peptide. D3D3 is a dimer of this peptide connected by a peptide bond between the N and C termini.

Methods

Animals

Our study used female, AD Tg mice (APPSweDI) divided into three groups: D3, D3D3, and control (saline). Female mice were chosen in order to maintain consistency with a previous

peptide study. The animals received 1 mg per month of D3 peptide, D3D3 peptide, or saline intraperitoneally over the course of one month (4.5-5.5 months of age) via an Alzet minipump (model 1004). The experiments were conducted in accordance with the local Institutional Animal Care and Use Committee (IACUC) guidelines.

Behavioral and cognitive analysis

The mice were analyzed in a battery of behavioral tests. To rule out differences in anxiety or stress levels, the open field and zero maze tests were applied. For the analysis of cognitive deficits, the mice underwent the Morris water maze test. During days 1 through 5 of the water maze testing period, the mice were trained to find a hidden platform that was kept in a constant position. Four trials per day were run so that all starting positions were used equally in a random order. The mice were given 60 s to find the platform and 10 s to stay on the platform. The inter-trial interval was approximately 2 min. Learning of the task was evaluated by recording the swimming speed, latency to find the platform, path length, and percentage of trials each animal found the platform. After the end of the four trials on day 5 of the testing period, the mice were tested in a 60 s probe trial (trial 21), with no escape platform present.

Mice that learned the platform position will predominantly search in the correct quadrant of the pool in the probe trial. All behavioral testing and statistical analysis of the behavioral data were performed in the UAB Behavioral Assessment Core.

Immunohistochemical and biochemical analysis

Following behavioral and cognitive analysis, the animals were anesthetized and perfused transcardially with a cold saline solution. The brain was dissected and the left hemisphere was frozen at −80 °C, then used for ELISA measurements of Abeta levels. The right hemisphere was fixed in 4% paraformaldehyde solution.

Histology

Brains were cut into 6 series of 30-mm sections (coronal plane). Sections were stained with amyloid β (WO2-antibody), glial fibrillary acidic protein (GFAP; astrocytes), and ionized calcium binding adaptor molecule 1 (Iba-1; microglia). Series 4 – 6 were stored for later analysis. Following incubation in the primary antibody for 18 h, the sections were rinsed in Tris-Buffered Saline with Tween and incubated with the appropriate biotinylated secondary antibody for 2 h. Following rinsing, the sections were put in a solution containing Extra-Avidin for 2 h, then rinsed and stained for approximately 3 min with Ni-enhanced diaminobenzidine. Stained sections were mounted on slides and coverslipped. Some sections of these series were counterstained with Congo red (Aβ plaques), mounted, and coverslipped.

Results

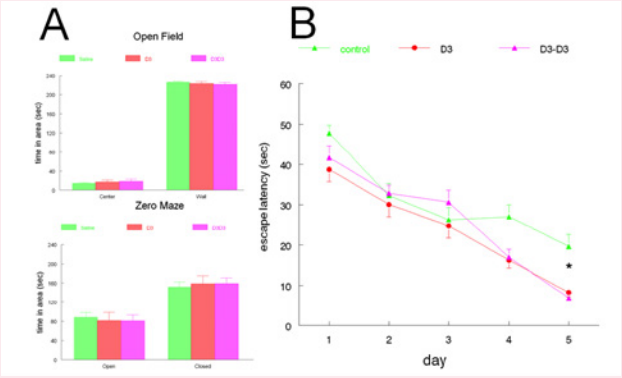

Figure 2. (A) Time spent in the different areas of the open field and elevated plus mazes. Animals did not show significant differences in anxiety or activity. (B) Learning curves of the mice in the water maze. D3 and D3D3 treated mice showed significant improvement compared with control.

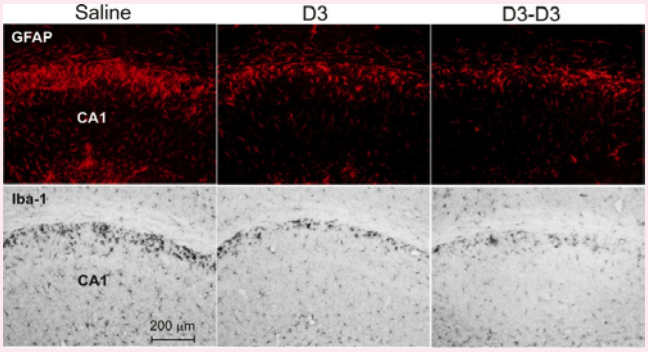

Figure 3. Photomicrographs of the dorsal hippocampus, stratum oriens. Upper panel: GFAP (astrocytes) stained sections; lower panel: Iba-1 (microglia) stained sections. Sections from peptide treated animals displayed decreased staining density.

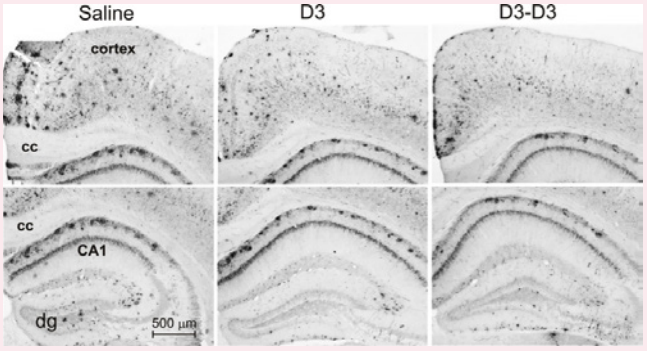

Figure 4. Photomicrographs of sections stained for human Aβ showing the dorsal, midline cortex and hippocampus. Sections from peptide treated animals displayed decreased staining density. Corpus callosum (cc) and dentate gyrus (dg) are shown.

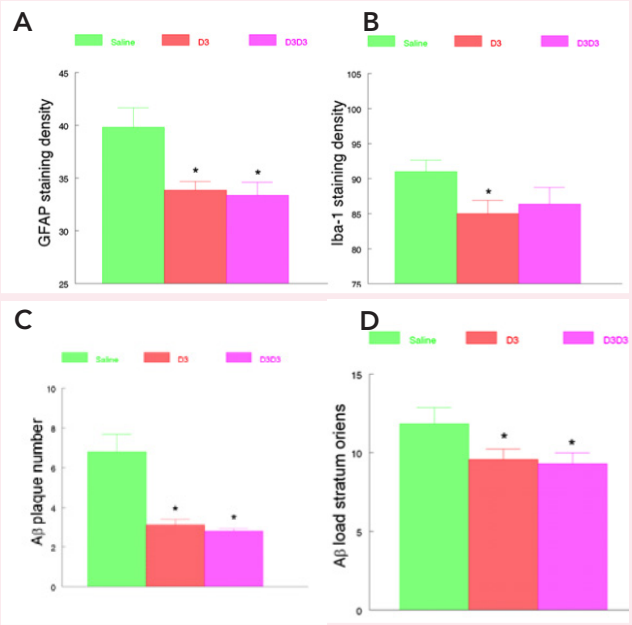

Figure 5. Quantification of the staining density of (A) GFAP, (B) Iba-1, (C) Aβ, and (D) Congo red. Staining density was significantly reduced in peptide-treated mice.

Tests for anxiety resulted in no difference between the control group and the two experimental groups (Figure 2A). Spatial learning and memory functioning was assessed by measuring escape latency in the water maze task. Animals in the D3 and D3D3 groups displayed significantly faster escape times by day five than those in the control group, indicating an improvement in cognitive functioning (Figure 2B). While a significant difference was seen in the learning curve, no significant difference was seen between groups in the probe trial (not shown graphically).

Figure 3 shows GFAP and Iba-1 staining of the dorsal hippocampus, specifically the CA1 region. The top section was stained with GFAP to detect astrocytes, which cluster around Aβ plaques. Significant reduction in astrocyte clusters can be seen in the D3 and D3D3 stains compared with the control region. The bottom panel was stained with Iba-1 to detect the presence of microglia, the immune cells of the nervous system. A significant reduction in microglia accumulation was seen in the D3 group but not in the D3D3 group compared with the control group. Figure 4 shows sections of the hippocampus stained for the presence of human Aβ. A significant reduction in plaque density can be seen in the D3 and D3D3 groups compared with the control group.

Figure 5 depicts graphical representations of the data presented in Figures 3 and 4 as well as results for Congo red staining (not pictorially represented). A significant reduction in the number of Aβ plaques observed was noted in the D3 and D3D3 groups compared with the control group. There was no significant difference in number of plaques between experimental groups.

Discussion

In this study, we intraperitoneally infused Tg AD model mice with one of two small D-amino acid peptides (D3 or D3D3) that have a high affinity for $A\beta_{42}$,[2-3] examined cognitive performance after 3 weeks, and measured the Aβ deposits in the hippocampus and cortex 4 weeks later. No difference in cognitive deficits was observed between D3- and D3D3-treated mice, but saline-treated mice were significantly impaired compared to the other two groups. The data demonstrate that short-term infusion with the D3 or D3D3 peptides leads to a reduction in the density of amyloid β deposits in the hippocampus and cortex, whereas infusion with the vehicle solution does not change amyloid β deposition patterns. Furthermore, analysis of glial stainings (GFAP and Iba1) shows significant reduction in both activated astrocytes and microglial cells surrounding Aβ plaques in both the D3 and D3D3 infused mice compared to the control mice.

One of the two pathological hallmarks of human AD is the presence of neuritic plaques.[4] Neuritic plaques have a dense core of aggregated Aβ peptides.[5] However, diffuse amyloid deposits have also been shown to be present in AD brains[4,6] and in the brains of AD model mice.[7] The data from our experiments show that both the D3- and D3D3-infused mice have a lower Aβ load, demonstrating that binding of the peptide to $A\beta_{42}$ reduces Aβ deposition in the hippocampus and neocortex. This is most likely due to changes in the aggregation properties of the $A\beta_{42}$-D peptide complex, lessening the propensity of $A\beta_{42}$ to aggregate.[8-9] This would lead to a decrease in the Aβ deposition rate by reducing either the number of plaques, the size of plaques, or both.[3] Conversely, it is possible that the clearance of the D3-$A\beta_{42}$ complex has improved compared to the clearance of $A\beta_{42}$.[10-12] Several mechanisms have been proposed for the clearance of Aβ, including receptor-mediated Aβ transport across the blood-brain barrier and enzyme-mediated degradation of the peptide.[10,12] It is more likely that Aβ transport out of the brain is improved than that Aβ degradation has increased, since the properties of the D3-$A\beta_{42}$ complex are not likely to improve enzymatic degradation.[13]

All plaques in our AD-model mice are accompanied by activated glial cells, both astrocytes and microglia.[14-15] The role of the activated glial cells is unclear; they could protect the brain by removing Aβ, or they could secrete inflammatory cytokines and generate NO, thus damaging and killing bystander neurons.[16] In our mouse model, no dead or dying neurons are present, even near plaques.[12] In the D3 and D3D3 infused mice, dense amyloid β deposits are associated with much lower numbers of activated microglia or activated astrocytes. This suggests that the binding of the D-peptides to deposited $A\beta_{42}$ changes the structure of $A\beta_{42}$, evoking fewer inflammatory responses.[9,13] Furthermore, the size of the Aβ deposits is smaller in both D-peptide-treated mice,

suggesting that the Aβ in the plaques, through binding with the D-peptide, exhibits lower aggregation kinetics. Finally, it should be noted that the number of deposits that stain for Congo red has also been significantly reduced. Either fewer new deposits were formed during the four weeks of treatment, or deposits were removed by microglia.

The data demonstrate that short-term infusion with the D3 and D3D3 peptides leads to a reduction in the density of amyloid β deposits in the hippocampus and cortex, whereas infusion with the vehicle solution does not. Cognitive deficits are not significantly different between control and D3-infused mice. Furthermore, the D3- and D3D3-infused mice demonstrate a significant reduction in both activated astrocytes and microglial cells surrounding Aβ plaques compared to the control mice. Together, this demonstrates that, while neither peptide is superior over the other, D3 and D3D3 have great potential as future treatments in Alzheimer's disease.

Conclusion

Treatment with both D-peptides improved cognitive functioning and significantly reduced Aβ load and inflammation in AD transgenic (APP/PS1) mice; however, D3D3 was not superior to D3. New peptides are currently being developed based on D3. We hope these new peptides will be more efficacious than D3 and/or D3D3.

A key demonstration of this study is that oral treatment with D3 and D3D3 peptides is able to improve cognitive deficits, decrease amyloid β pathology, and decrease inflammation. These findings suggest possible future use of these peptides toward treatment of Alzheimer's disease in humans.

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