Targeting Age-related Macular Degeneration With Alzheimer’s Disease Based Immunotherapies: Anti-Amyloid-β Antibody Attenuates Pathologies in an Age-related Macular Degeneration Mouse Model

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Abstract

Age-related macular degeneration (AMD) is a late-onset, neurodegenerative retinal disease that shares several clinical and pathological features with Alzheimer’s disease (AD) including extracellular deposits containing Amyloid beta (Aβ) peptides. Immunotherapy targeting the Aβ protein has been investigated as a potential treatment for AD. Here, we present the rationale for extending this approach to treat AMD. We tested an anti-Aβ antibody administered systemically in a mouse model of AMD. Histological and functional measurements in treated animals compared to controls showed that following immunotherapy, the amounts of Aβ in the retina and brain were decreased and the ERG deficits in the retina were attenuated. These data support the hypothesis that Aβ is a therapeutic target for AMD.

Keywords

Age-related macular degeneration (AMD); amyloid; choroidal neovascularization (CNV); retinal pigment epithelium (RPE); immunotherapy

1. Age-related Macular Degeneration

Age-related macular degeneration (AMD) is a late-onset neurodegenerative retinal disease that manifests as progressive loss of central vision due to dysfunction and death of photoreceptor and adjacent retinal pigment epithelial (RPE) cells in the macula. It is the leading cause of irreversible vision loss in people over the age of 65 (Javitt, Zhou et al. 2003). AMD is a multifactorial disease in which the relative contributions of genetic and environmental factors...
remain unknown. The strongest known risk factor for AMD is advanced age, however, other identified risk factors include gender, ethnicity, smoking, hypertension, hypercholesterolemia, and diet (van Leeuwen, Klaver et al. 2003). It is now established that AMD is also influenced by genetic factors, as evidenced by identification of AMD-susceptibility genes (Piguet, Wells et al. 1993; Heiba, Elston et al. 1994; Klein, Mauldin et al. 1994; Silvestri, Johnston et al. 1994; Evans and Bird 1996; Klein, Schultz et al. 1998). One gene implicated is \textit{APOE}, a polymorphic gene in humans encoding one of 3 common alleles (\(\epsilon_2\), \(\epsilon_3\) and \(\epsilon_4\)) with the \(\epsilon_4\) allele associated with AMD (Baird, Guida et al. 2004). Apolipoprotein E (apoE) is an amphipathic glycoprotein that mediates the distribution of lipids and cholesterol among cells. A Y402H polymorphism in another gene, \textit{CFH}, coding for complement factor H and factor H-like protein 1, was recently shown to be the major genetic factor contributing to AMD (Edwards, Ritter et al. 2005; Hageman, Anderson et al. 2005; Haines, Hauser et al. 2005; Klein, Zeiss et al. 2005).

2. Mouse model of AMD

We developed a murine model of AMD that combines three known risk factors for AMD: advanced age, apoE isoform expression and exposure to a high-fat, cholesterol-enriched (HF-C) diet (Malek, Johnson et al. 2005). Specifically, mice carrying a targeted knock-in of the human \textit{APOE} \(\epsilon_4\) (\textit{APOE4}) gene, aged over 1 year and fed a HF-C diet for 8 weeks develop pathological features similar to the morphologic hallmarks observed in both dry and wet human AMD. Importantly, these changes require the presence of all three risk factors. These changes were not detected in any of the control, human \textit{APOE3} expressing mice regardless of diet consumed, nor were there any pathologies detected in young \textit{APOE4} animals. This animal model of AMD with spontaneously-occurring CNV is the first to incorporate physiologically-relevant risk factors of human disease and therefore can be used to rationally identify and test therapeutic targets for intervention. To date, the results of our studies using this model suggest that lipid transport dysregulation and amyloid deposition contribute to the pathogenesis of the observed retinal changes (Malek, Johnson et al. 2005).

3. Alzheimer’s disease, amyloid and A\(\beta\)

Like AMD, AD is a multifactorial disease affected by age, environmental factors and genetics. In AD the major genetic risk factor is \textit{APOE}. The common \textit{APOE} gene polymorphisms are linked to 90% of cases < 65 years of age and 60% of AD cases > 65 years. Whereas the function of apoE in the central nervous system remains unclear, apoE functions in the periphery by maintaining cholesterol and triglyceride homeostases (Mahley 1988). The pathological hallmarks of AD include the presence of senile plaques, neurofibrillary tangles, neuronal cell loss, reactive gliosis and, in some cases, cerebrovascular amyloid deposits (reviewed by Blennow, de Leon et al. 2006). Senile plaques are generated by deposition and accumulation of the amyloid beta (A\(\beta\)) peptides. Cholesterol has been linked to amyloidogenesis, and there is increasing evidence of the importance of cholesterol as a risk factor for AD through both intracellular and extracellular mechanisms (Canevari and Clark 2007).

A\(\beta\) peptides are derived from amyloid precursor protein (APP) through sequential, proteolytic cleavage at the N-terminus by \(\beta\)-secretase and at the C-terminus by \(\gamma\)-secretase. The \(\gamma\)-secretase is unique among proteases in that it cleaves APP in the transmembrane region, resulting in a peptide of 40 or 42 amino acids in length (A\(\beta1–40\) or \(1–42\)). After A\(\beta1–40\) or \(1–42\) peptides are generated, its N-terminus can be further modified or cleaved, yielding N-terminal truncated peptides A\(\beta\)x-40 or A\(\beta\)x-42 (Arai, Akiyama et al. 1999). Mutations of APP and presenilins (PS1 and PS2, integral components of the gamma-secretase protease complex) have been identified in familial forms of AD. APP-overexpressing and APP/PS1 double transgenic mice
exhibit brain deposits of amyloid (plaques) and accompanying memory impairment, underscoring the notion that accelerated Aβ peptide production is sufficient to lead to AD.

4. Aβ Immunotherapies

Immunotherapies targeting the Aβ peptide, using vaccines or antibodies, have been applied to several different strains of APP-based mouse models of AD to reduce amyloid plaques and improve cognitive performance (Hardy and Selkoe 2002). Although promising, phase 2 clinical trials of the first generation Aβ vaccine AN-1792 (Elan/Wyeth) were halted after a significant number of patients (6%) developed autoimmune meningoencephalitis characterized by perivascular infiltration, due primarily to brain infiltration of autoreactive T lymphocytes in response to active immunization (Nicoll, Wilkinson et al. 2003; Orgogozo, Gilman et al. 2003; Ferrer, Boada Rovira et al. 2004). Importantly, unlike typical AD patients, the AN-1792 treated AD patients undergoing autopsy were found to have extremely low amyloid burdens in their cerebral cortex, suggesting the tantalizing possibility that the vaccine was, in fact, effective in removing the amyloid plaques (Nicoll, Wilkinson et al. 2003; Masliah, Hansen et al. 2005).

To reduce the risk of uncontrolled CNS inflammation associated with active vaccines, passive immunization using a monoclonal antibody targeting the N-terminus (Bapineuzumab, Elan/Wyeth) or the middle portion (LY2062430, Eli Lilly) of the Aβ peptide are now under investigation in phase 2 clinical studies (Melnikova 2007). However, as mentioned above, the N-terminal portion of Aβ peptides are often truncated, rendering the monoclonal antibodies targeting the N-terminus ineffective for these truncated Aβ peptides in the AD brain. On the other hand, antibodies recognizing the middle portion of the Aβ peptides can also bind the full length APP protein normally found on the cell surface in the CNS, creating a potential safety risk for neurons and other healthy APP expressing cells in patients.

Therefore, we turned our attention to the novel therapeutic approach of targeting Aβ peptides at the C-terminus. These C-terminal epitopes are normally buried in the lipid bilayer and are exposed, predominantly, in the pathological state. Two different C-terminal x-40 specific monoclonal antibodies, 2286 and 2H6, were identified and tested in Tg2576 APP-overexpressing mouse model (Wilcock, Rojiani et al. 2004). Both antibodies, given either intracranially or peripherally, were effective in reducing brain levels of p. 5 amyloid plaques and improving cognitive behaviors. The peripheral administration of these C-terminal x-40 specific antibodies was also accompanied by a 10–100 fold increase in circulating Aβ peptide levels, supporting the notion that Aβ antibodies in the periphery create a sink, pulling soluble brain Aβ into the blood for clearance. Since both 2286 and 2H6 antibodies have native mouse immunoglobulin constant regions that can mediate immune effector functions, such as binding to Fcγ receptors, complement fixation and antibody-dependent cell-mediated cytotoxicity (ADCC), we sought to further reduce the potential safety risk of C-terminal specific Aβ antibodies. In previous studies, deglycosylated 2H6 (i.e. 2H6-D) did not bind any Fcγ-I receptors, complement or mediate any detectable ADCC. The administration of 2H6-D in Tg2576 mice, while still effective in ameliorating the AD pathology and behavioral deficits, was associated with significantly lower levels of microhemorrhages and inflammation in the brain than the parent antibody, 2H6 (Carty, Wilcock et al. 2006; Wilcock, Alamed et al. 2006).

5. Rationale for Aβ immunotherapy in AMD

As mentioned above, AD and AMD share several striking pathological features in common. First and foremost, the risk of AD and AMD both increase dramatically with advanced age. Furthermore, they are both associated with pathological, extracellular deposits consisting of Aβ peptides, apoE protein, complement and other components.
In human AMD, Aβ deposition was associated with drusen from AMD eyes, but not with drusen in normal eyes (Dentchev, Milam et al. 2003), where it accumulates and co-localizes with activated complement components within drusen (Johnson, Leitner et al. 2002; Anderson, Talaga et al. 2004). Likewise, we detected Aβ peptide in aged APOE4 mice exposed to a HF-C diet (APOE4-HFC) but not in control animals (Malek, Johnson et al. 2005). In addition, human CNV membranes and neovascular (NV) tissue from APOE4-HFC mice display perivascular Aβ immunoreactivity, whereas Aβ immunoreactivity was also detected in subRPE deposits in our AMD model (Malek, Johnson et al. 2005). A pathogenic role for amyloid in AMD was proposed by Yoshida et al. in studies showing that Aβ increases VEGF mRNA and protein levels in primary cultures of human RPE cells, (Yoshida, Ohno-Matsui et al. 2005), and evidence of amyloid toxicity was shown by Luibl et al. who detected nonfibrillar oligomeric Aβ in drusen from AMD eyes (Luibl, Isas et al. 2006).

Based on (1) the detection of amyloid in both our animal model and in AMD patients, (2) the findings that APOE genotype regulates Aβ cytotoxicity and (3) the considerable overlap between the pathologies of AMD and AD, we tested the hypothesis that Aβ is a toxic, pathogenic component of basal deposits by using an anti-Aβ antibody to deplete Aβ in a mouse model of AMD to determine if this could ameliorate the disease.

6. Pilot Study of anti- Aβ in AMD mouse

6.1 Methods

Animals—Mice were maintained and bred in accordance with the Institutional Animal Care and Use Committee at Duke University and the Association for Research in Vision and Ophthalmology statement for the use of animals in ophthalmic and vision research. APOE4 targeted replacement (TR) mice expressing the human apoE4 isoform under control of the endogenous mouse apoE regulatory sequences, were generated as previously described (Sullivan, Mezdour et al. 1997).

Experimental protocol, diet and antibody administration paradigm—A small cohort of aged male (n=19; 72–120 weeks) C57Bl/6 APOE4 TR mice housed conventionally, under ambient conditions were switched from normal mouse chow (ND, Isopurina #5001; Prolab, Dewitt, NY) to a high fat diet rich in cholesterol (HF-C, #T.D. 88051; Harlan Teklad, Indianapolis, IN) for 8 weeks immediately prior to analysis as previously described (Malek, Johnson et al. 2005). Age-matched normal control APOE4 TR littermates (“APOE4-ND”, n=3) were fed ND and water ad libitum. The HF-C fed APOE4 mice (APOE4-HFC) were assigned to one of 3 groups based on antibody treatment as follows:

1. Uninjected controls (n=3), “APOE4-HFC-uninj”, received no injections.
2. Vehicle injected controls (n=8), “APOE4-HFC-PBS”, received 1X per week intraperitoneal (IP) injections of vehicle (phosphate buffered saline, PBS) for a period of 8 weeks coinciding with HF-C diet.
3. Anti-Aβ injected (n=8), “APOE4-HFC-Anti-Aβ”, received 1X per week IP injections of 3 mg/kg body weight/injection of de-2H6 (deglycosylated anti-Aβ33–40; IgG2b isotype; Rinat Neurosciences, South San Francisco, CA) which recognizes both mouse and human Aβ (Wilcock, Rojiani et al. 2004; Wilcock, Alamed et al. 2006).

Antibodies, antibody doses and injection paradigm used were based on parameters established by previously in AD mouse models (Wilcock, Alamed et al. 2006). At the end of the diet/injections exposure, mice were euthanized with an overdose of aversin and flushed with a 20ml bolus of saline.
**Electroretinogram (ERG) Recordings**—The ERG recordings were obtained from animals, dark adapted for at least 12 hours prior to recording. Each animal was anesthetized with a ketamine/xylazine cocktail, pupils were dilated and after the animal stabilized on a 37°C warming pad, ERG tracings were recorded using a silver fiber test electrode placed in contact with the eye along with a drop of 2.5% hydroxypropyl methylcellulose. Each mouse was exposed to flashes of light (max intensity of 1000 cd-s/m² attenuate in 1 log steps, starting from 0.0005). The a-wave amplitude was measured from baseline to the a-wave trough, and the b-wave amplitude was measured from the a-wave trough to the b-wave peak.

**Histology and Immunohistochemistry**—Eyes were immersion-fixed in 4% paraformaldehyde for 2 hours and transferred to 2% paraformaldehyde and stored at 4°C until processing for light microscopy. The anterior segment was removed and posterior segment was cryo-protected with serial sucrose (10%, 20% and 30%) solution. Frozen sections were cut on a cryostat at ~10 μm. Sections were treated with 3% H₂O₂ for 30 min, preincubated with 10% BSA, and incubated overnight with biotinylated monoclonal mouse anti-Aβ antibody (1:100; clone 4G8, Signet). The signal was then amplified using a TSA biotin kit (PerkinElmer), following the manufacturer’s instructions. Sections were then incubated with streptavidin-conjugated Alexa Fluore 594, to visualize the signal. For quantification, 3 to 4 images were randomly taken for each of 5 antibody-treated and 5 saline-treated animals. Aβ-positive puncta in basal deposits and BM were counted, and the length of BM was also measured. Results are presented as linear density along BM.

The brain was immersion fixed in 10% buffered formalin (4% formaldehyde, pH 7.4) for 18 hours at 4°C, prior to being transferred to 1xPBS, pH 7.4, 4°C. The brain was then sectioned on an Oxford vibratome at 30 μm then reacted as free-floating sections. Aβ immunohistochemistry (IHC) was performed with the biotin-avidin-peroxidase method, using diaminobenzidine (DAB) as a chromogen (Vector Laboratories, CA). All tissue sections including controls are run simultaneously to avoid inter-run variability. No antibody was used for visualizing background reactivity for negative control for each animal. IHC tissue is pretreated in 10% methanol and 2% peroxide in PBS and then 88% formic acid for 1 min. After blocking in serum, the tissue is incubated with a biotinlyated mouse anti-human Aβ monoclonal (1:2000) (4G8, Senetek, Napa, CA) at room temperature, 2hrs. The tissue is then incubated with an avidin-biotin conjugate ABC reagent, as manufacture instructions (Vector Laboratories, CA). DAB (1.39 mM) in 1X PBS, 0.01% peroxide is used as the chromogen for visualization of staining. No secondary staining was used. Stained slides were examined under light microscopy (Nikon optiphot-2). In order to eliminate any bias in analyzing the tissue, several pictures from each section were digitally captured by a cooled CCD camera Penguin 660cl (Pixera).

### 6.2 Results

**ERG Recordings**—We have previously shown that aged APOE4 animals following exposure to the HF-C diet for 8 weeks (APOE4-HFCs) develop AMD-associated ocular pathologies (Malek, Johnson et al. 2005), and that in these animals there was statistically significant reduction in the a- and b-wave amplitudes compared to age-matched, histologically normal control APOE4 mice maintained on the normal rodent chow diet, APOE4-ND (Malek, Jamison et al. 2007). In the current pilot cohort of aged APOE4-HFC mice that were either un.injected or received 8 weekly IP injections of the saline-control a similar decrease in the b-wave amplitudes was observed compared to the normal APOE4-ND (Figure 1). In contrast, there was a preservation of the b-wave amplitude in the APOE4-HFC mice that received 8 weekly IP injections of the anti-Aβ antibody (blue trace, Figure 1). A similar trend was observed in the a-wave amplitudes though it was not as robust (data not shown).
Amyloid immunohistochemistry—Consistent with the improvement of ERG, total Aβ immunostaining in the retina of the E4-HFC mice was reduced after 8 weeks immunotherapy with antibody 2H6-D as compared to the control vehicle group, as illustrated in Figure 2. On average, the control saline-treated group had 116 puncta per 1 mm of BM, whereas anti-Aβ antibody-treated group had 41 (Figure 2; p = 0.048, one-tailed t-test). Similarly, the diffuse amyloid plaques revealed by the total Aβ immunostaining in the cerebral cortex of the E4-HFC mice were also greatly reduced after 8 weeks immunotherapy with antibody 2H6-D relative to the control vehicle group (Figure 3). Vascular amyloid in the brain was more prevalent in untreated E4-HFC animals (not shown).

These preliminary findings demonstrate 1) preservation of retinal function as demonstrated by ERGs of mice injected with the anti-Aβ antibody, 2H6-D and 2) reduction of amyloid deposition when treated with anti-Aβ antibody in mouse retina and cortex as compared to the untreated AMD mouse group.

7. Summary and Future Directions

Despite the shared similarities between AD and AMD, AMD has thus far not been classified as an amyloid disease. Among the principal differences is the fact that classical amyloid diseases typically exhibit large amounts of amyloid fibrils (Soto 2003). For example, in the case of AD, the characteristic plaques consist primarily of fibrillar Alzheimer Aβ peptide. Amyloid fibrils display characteristic tinctorial properties, such as thioflavin T and congo red staining (Puchtler and Sweat 1965; LeVine 1999; Krebs, Bromley et al. 2005). Though drusen do stain with thioflavin T and congo red, the characteristic birefringence often seen in congo red–stained amyloid fibrils is not present (Mullins, Russell et al. 2000; Anderson, Talaga et al. 2004).

Even though AMD is not widely considered as a prototypical amyloid disease, previous studies from several laboratories, including ours, have localized Aβ peptide-derived amyloid within subRPE deposits and CNV in human AMD eyes and our murine model implicate Aβ amyloid in AMD pathogenesis. This is further supported by our pilot immunotherapy study targeting Aβ described above.

These findings, though preliminary, contribute to understanding underlying mechanisms of pathology specifically related to the role of amyloid in sub-RPE deposit formation and retinal changes in AMD. These experiments support the feasibility of immunotherapeutic strategies targeting amyloid as treatments for AMD. Finally, our pilot data also suggest the exciting prospect that, in the aging population, two of the most common neurodegenerative diseases, AD and AMD, could simultaneously be treated or prevented with a single therapeutic agent, such as the anti-Aβ antibody.

Acknowledgements

The authors gratefully acknowledge Drs. Goldis Malek and Dennis Rickman for scientific discussions and the following funding agencies: NEI P30 EY005722, NEI R21 EY01712 (CBR), A Ruth and Milton Steinbach Award (CBR), Pfizer Pilot funds (CBR) and Research to Prevent Blindness Core Grant.

References


Vision Res. Author manuscript; available in PMC 2009 February 1.
Figure 1. 

b-wave in aged APOE4 mice fed a normal or HF-C diet and in aged APOE4-HFC’s injected with anti-Aβ antibody (HFC-αAβ) or with saline vehicle control (HFC-con). V-log I intensity response curves of the b-waves. Baseline ERGs (n=12 to 14) obtained from APOE4-ND controls (black) and APOE4-HFC controls (red). E4-HFC-con vehicle injected (green), E4-HFC-αAβ (blue). Data are plotted with SE bars. The b-wave in the antibody-treated animals is preserved compared to the saline-treated group (p < 0.2, equal variance 2-tailed T-test).
Figure 2.
Amyloid detected in APOE4 mouse retina using 4G8 anti-Aβ antibody (A–C) and quantification (D). A: APOE4-ND, B: APOE4-HFC vehicle injected control, C: APOE4-HFC injected with anti-Aβ antibody, de-2H6. Note positive Aβ staining (red) along Bruch’s membrane (BM) in APOE4-HFC vehicle injected control retina (arrowheads in B), but not in antibody treated APOE4-HFC mouse retina (C). Lipofuscin autofluorescence: bright yellow puncta (A, C and E). D: Summary and statistical results of Aβ linear density along BM. Black dots represent average Aβ density for individual animals. Bars and error bars show averaged density and standard deviation. The linear density of Aβ-positive puncta of antibody-treated group is significantly reduced compared to saline-treated group (p = 0.048, one-tailed t-test).
Figure 3.
Amyloid in mouse brain (cortex) using 4G8 anti-Aβ antibody. Amyloid deposits decreased in aged APOE4-HFC treated with anti-Aβ-antibody, de-2H6. A,C: Representative sections through the cortex of apoE4 treated mice stained with an anti-amyloid beta antibody (4G8) showing almost no amyloid even in the vascular regions of the tissue. B,D: Control aged apoE4 mice on HF-C diet showing an occasional parenchymal amyloid deposit. E: Negative control with no antibody was used to measure background staining. F: human transgenic PdAPP mouse tissue (a mouse that deposits human amyloid in the brain) was used for amyloid positive control (gift from Kelly Bales, Eli Lilly).