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Receptor-Mediated Abeta Amyloid Antibody Targeting to Alzheimer's Disease Mouse Brain

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Abstract: The goal of this work is the reduction in the Abeta amyloid peptide burden in brain of Alzheimer's disease (AD) transgenic mice without the concomitant elevation in plasma Abeta amyloid peptide. An anti-Abeta amyloid antibody (AAA) was re-engineered as a fusion protein with a blood−brain barrier (BBB) molecular Trojan horse. The AAA was engineered as a single chain Fv (ScFv) antibody, and the ScFv was fused to the heavy chain of a chimeric monoclonal antibody (mAb) against the mouse transferrin receptor (TfR), and this fusion protein was designated cTfRMAb-ScFv. The cTfRMAb-ScFv protein penetrates mouse brain from blood via transport on the BBB TfR, and the brain uptake is 3.5% of injected dose/gram brain following an intravenous administration. Double transgenic APPswe,PSEN1dE9 mice were studied at 12 months of age. The mice were shown to have extensive Abeta amyloid plaques in cerebral cortex based on immunocytochemistry. The mice were treated every 3−4 days by intravenous injections of either saline or the cTfRMAb-ScFv fusion protein at an injection dose of 1 mg/kg for 12 consecutive weeks. The brain Aβ^{1-42} concentration was reduced 40% in the fusion protein treated mice, without any elevation in plasma Aβ^{1-42} concentration. No cerebral microhemorrhage was observed in the treated mice. These results show that brain-penetrating antibody pharmaceutics can be developed for brain disorders such as AD following the re-engineering of the antibody as a fusion protein that is transported across the BBB via receptor-mediated transport.

Keywords: Blood−brain barrier; drug targeting; Alzheimer's disease; monoclonal antibody; amyloid

Introduction

The dementia of Alzheimer's disease (AD) correlates with the deposition in brain of amyloid.[1,2] AD amyloid is composed of the Abeta peptide,[3] which is derived from the abnormal processing of the amyloid peptide precursor (APP) protein in brain.[4] The intracerebral injection of an anti-amyloid antibody (AAA) results in the rapid disaggregation of amyloid plaque, which is associated with the repair of dystrophic neurites.[5,6] The passive immune therapy of AD is designed to administer AAAs systemically to patients with AD in an attempt to cause disaggregation of the brain amyloid.[7−9] The AAA-mediated disaggregation of amyloid plaque requires the physical interaction between the plaque

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and the AAA.[10] The amyloid plaque resides in brain, behind the blood−brain barrier (BBB). However, AAAs are large molecule pharmaceuticals that do not cross the BBB.[11] Therefore, AAAs cannot penetrate the brain from blood in the absence of BBB disruption. BBB disruption may be the mechanism by which AAA administration causes disaggregation of amyloid plaque in the brains of AD transgenic mice.[12] AAA administration is associated with cerebral microhemorrhage in brain of AD mice,[13,14] which is associated with large increases in plasma concentration of the Abeta amyloid peptide.[14] The cerebral microhemorrhage observed in mice treated with AAA therapy correlates with the vasogenic edema associated with AAA therapy in humans with AD.[15] An alternative form of AAA therapy of AD is an AAA that is re-engineered to penetrate the BBB in the absence of BBB disruption, as well as to undergo rapid removal from the blood, so as to avoid elevations in plasma Abeta peptide concentrations.

AAAs can be re-engineered to both cross the BBB from blood and rapidly exit the blood via receptor-mediated transport by fusion of the AAA to a BBB molecular Trojan horse.[11] The latter is a peptide or peptidomimetic monoclonal antibody (mAb) against an endogenous receptor-mediated transporter on the BBB. The most active BBB molecular Trojan horse is a genetically engineered mAb against the human insulin receptor (HIR). A fusion protein between an AAA and the HIRMAb has been engineered and has been shown to both rapidly penetrate the Rhesus monkey brain from blood and rapidly exit the plasma compartment.[11] The HIRMAb-AAA fusion protein disaggregated brain amyloid plaque in AD transgenic mice following intracerebral injection.[11] It was necessary to inject the HIRMAb-AAA fusion protein into the brain in mice, because the HIRMAb part of the fusion protein does not bind to the insulin receptor in species other than humans or Rhesus monkeys.[16] There is no known mAb against the murine insulin receptor that can be used as a BBB Trojan horse in the mouse. A surrogate molecular Trojan horse that is active in the mouse is a genetically engineered chimeric mAb against the mouse transferrin receptor (TfR), which is designated the cTfRMAb.[17] A fusion protein of the cTfRMAb and a single chain Fv (ScFv) antibody, which was produced with variable regions derived from an AAA, has been engineered and expressed, and is designated the cTfRMAb-ScFv fusion protein.[18] The cTfRMAb-ScFv fusion protein binds the BBB TfR and rapidly enters the mouse brain from blood.[18] The brain uptake of the cTfRMAb-ScFv fusion protein in the mouse is 3.5 ± 0.7% of injected dose (ID) per gram brain at 60 min following intravenous (iv) administration.[18] This brain uptake of the cTfRMAb-ScFv fusion protein is high compared to the brain uptake of a mAb that does not cross the BBB in the mouse, which is 0.06% ID/g.[19] In addition, the cTfRMAb-ScFv fusion protein accesses the TfR in

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peripheral tissues, and is rapidly removed from the blood with a mean residence time (MRT) of less than 3 h in the mouse.[18]

The goal of the present study was to test the feasibility of reducing the Abeta amyloid content in the brain of AD transgenic mice without a concomitant elevation in plasma Abeta peptide. The cTfRMAb-ScFv fusion protein is administered to 12 month old AD transgenic mice by twice a week iv injection at a treatment dose of 1 mg/kg for a 3 month period. Brain and plasma Aβ^{1-42} concentrations were measured at the end of the study. This study also quantifies the development of an immune response to the cTfRMAb-ScFv fusion protein following chronic administration.

Experimental Section

Treatment of AD Transgenic Mice. AD transgenic mice were purchased from Jackson Lab (Bar Harbor, ME). The hemizygous male mice (strain APPswe, PSEN1dE9,[20] stock 305854) were 11 months old at the start of the 3-month study. The cTfRMAb-ScFv fusion protein was produced in stably transfected Chinese hamster ovary (CHO) cells and purified by protein G affinity chromatography, as described previously.[18] The mice (4 in each of the 2 treatment groups) were treated with either saline or the cTfRMAb-ScFv fusion protein (1 mg/kg) every 3−4 days via tail vein injection for 12 consecutive weeks or a total of 24 injections. Mice were euthanized, and the brain was removed for measurement of immunoreactive Aβ^{1-42} by ELISA, for Abeta immunocytochemistry (ICC) and for assessment of cerebral microhemorrhage by Prussian blue staining of mouse brain. Terminal plasma was examined for the presence of immune reactions against the fusion protein.

Aβ^{1-42} ELISA. At the end of the treatment, mice were euthanized, and the brain was removed and the left cerebral hemisphere homogenized in 10 volumes of homogenization buffer (5 M guandinine HCl, 0.05 M Tris, pH = 8.0) with a Polytron homogenizer followed by brief sonication and shaking at room temperature (RT) for 3 h. The homogenate was centrifuged at 10000*g* for 15 min at RT, and the supernatant was transferred to new tubes and stored at −20 °C. Prior to freezing of the homogenate, a 50 μL aliquot was removed and diluted to 2.5 mL in dilution buffer (0.02 M Tris, 0.15 M NaCl, 1 mM EDTA, 1% Triton X-100, pH = 7.5), and an aliquot was removed for protein assay using bicinchoninic acid (BCA) from Pierce Chemical Co. (Rockford, IL). The diluted homogenate was stored at −20 °C until ELISA. For Aβ^{1-42} ELISA, the sample was diluted 40-fold in TBS (0.02 M Tris, 0.15 M NaCl, pH = 7.5) for measurement of the immunoreactive Aβ^{1-42} by sandwich ELISA using a kit from Invitrogen (#KHB3441 or KHB3442). The standard curve was linear between 15.6 and 1000 pg/mL Aβ^{1-42}. The dilution buffer alone produced no signal above background in the ELISA. Mouse plasma samples were assayed with the Aβ^{1-42} ELISA after dilution in 1:10 in PBS (0.01 M Na2HPO4, 0.15 M NaCl, pH = 7.0).

Brain Microscopy. The right cerebral hemisphere without cerebellum was placed in cold 4% paraformaldehyde in 0.1 M KH_2PO_4/7.0 for immersion fixation at 4 °C for 24 h. The tissue was cryoprotected in 10%, 20%, and 30% sucrose in PBS at 4 °C for 24 h at each concentration of sucrose. The tissue was embedded in Tissue-Tek OCT medium and frozen in powdered dry ice, and 10 μm frozen sections were prepared on a cryostat at −15 °C. The sections were subbed to glass slides, and fixed in 4% paraformaldehyde for 20 min at 4 °C. For the immunocytochemistry, the slides were blocked with 0.1% H_2O_2/0.3% horse serum, and labeled with 2 μg/mL of biotinylated mouse monoclonal antibody against Aβ^{1-16} (Covance SIG-39340) overnight at 4 °C. Following washing, the slides were reacted with avidin and biotinylated peroxidase (Vectastain ABC, Vector Laboratories, Burlingame, CA) for 30 min at RT, color development was performed with Impact 3-amino-9-ethylcarbazole (AEC) substrate (Vector Laboratories), and the slides were counterstained with Mayer's hematoxylin. For the Prussian Blue tissue stain for brain microhemorrhage, the fixed slides were treated with 2% potassium ferrocyanide in 2% HCl in Coplin jars for 30 min at RT. Slides were counterstained with Mayer's hematoxylin.

Immunity ELISA. The presence of anti-cTfRMAb-ScFv fusion protein antibodies in mouse plasma was detected with a 2-site sandwich ELISA, using the cTfRMAb-ScFv fusion protein as the capture reagent and biotinylated cTfRMAb-ScFv fusion protein as the detector reagent. Alternatively, the cTfRMAb, mouse IgG1k, or the rat 8D3 mAb against the mouse TfR was used as the capture reagent. The cTfRMAb was engineered from the variable regions of the 8D3 mAb and the constant regions of the mouse IgG1 heavy chain and the mouse kappa light chain.[17] The mouse plasma was diluted in PBS. The capture reagent was plated overnight at 4 °C in 96 wells at 100 μL (250 ng)/well in 0.05 M $NaHCO_3$/8.3. The wells were blocked with PBS containing 1% bovine serum albumin (PBSB), followed by the addition of 100 μL/well of the diluted mouse plasma. After a 60 min incubation at 37 °C, the wells were washed with PBSB, and the wells were incubated with biotinylated cTfRMAb-ScFv fusion protein (10 ng/well) for 60 min. The wells were washed with PBSB, followed by incubation with 100 μL (500 ng/well) of a streptavidin−peroxidase conjugate (#SA-5004, Vector Laboratories) for 30 min at RT. The wells were washed with PBSB, and 100 μL/well of *o*-phenylenediamine/H_2O_2 developing solution (#P5412, Sigma) was added for a 15 min incubation in the dark at RT. The reaction was stopped by the addition of 100 μL/well of 1 M HCl, followed by the measurement of absorbance at 492 and 650 nm. The A650 was subtracted from the A492. The (A492 − A650) for the PBSB blank was then subtracted from the (A492 − A650) for the sample. Mouse plasma samples were screened with the immunity ELISA at 1:50 dilutions in PBS using the cTfRMAb-ScFv fusion protein as the capture reagent.

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For subsequent studies, plasma was pooled from the 2 fusion protein-treated mice that reacted strongest in the ELISA at 1:50 dilution. This pool was then diluted 1:50, 1:300, 1:1000, or 1:3000 in PBS, and the dilution curves were compared with 3 different capture reagents: the CHO-derived cTfRMAb,[17] the hybridoma-derived rat 8D3 mAb against the mouse TfR,[19] or mouse IgG1k, which is the isotype control for the constant region comprising the cTfRMAb.[17]

The cTfRMAb-ScFv fusion protein was biotinylated as described previously,[21] using sulfo-biotin-LC-LC-*N*-hydroxysuccinimide, where LC = long chain (#21338, Pierce Chemical Co., Rockford, IL). The biotinylation of the cTfRMAb-ScFv fusion protein was confirmed by SDS−PAGE and Western blotting, where the blot was probed with avidin and biotinylated peroxidase. The nonbiotinylated cTfRMAb-ScFv fusion protein gave no reaction in the Western blot, whereas the biotinylated protein was strongly visualized at the appropriate molecular size for both heavy chain and light chain.

Results

The Abeta immunocytochemistry of mouse brain showed that the AD transgenic mice developed extensive amyloid plaques (Figure 1A). Plaques were detected in cortex as well as subcortical structures such as the hippocampus. The concentration of immunoreactive Aβ^{1-42} in the brain was high, 125 ± 14 ng/mg$_p$ (Table 1). Treatment with the cTfRMAb-ScFv fusion protein caused a 40% reduction in brain immunoreactive Aβ^{1-42} to 75 ± 8 ng/mg$_p$ (Table 1), which was significant at the $p < 0.02$ level (Student's t test). The plasma immunoreactive Aβ^{1-42} was <150 pg/mL in both treatment groups (Table 1). Prussian blue staining of mouse brain in either treatment group showed no cerebral microhemorrhage in serial sections of cortex (Figure 1B) or hippocampus.

No mice developed signs of immune reactions, and no mice lost body weight during the course of the 12-week treatment study. The body weight of the saline treated mice was 34.4 ± 1.7 and 35.1 ± 0.6 g at the beginning and the end of the study. The body weight of the fusion protein treated mice was 31.9 ± 0.6 and 31.6 ± 0.6 g at the beginning and the end of the study. The design of the immunity ELISA is shown in Figure 2A. No immune response against the cTfRMAb-ScFv fusion protein was detected in the saline treated mice, whereas a variable immune response was detected with 1:50 dilutions of plasma obtained from mice treated with the cTfRMAb-ScFv fusion protein (Figure 2B). The immune response was primarily directed against the variable regions of the cTfRMAb, as shown by the dilution curves obtained with either the cTfRMAb or the rat 8D3 mAb as the capture reagent (Figure 2C). There was no immune response directed against the constant region of mouse IgG1κ (Figure 2C).

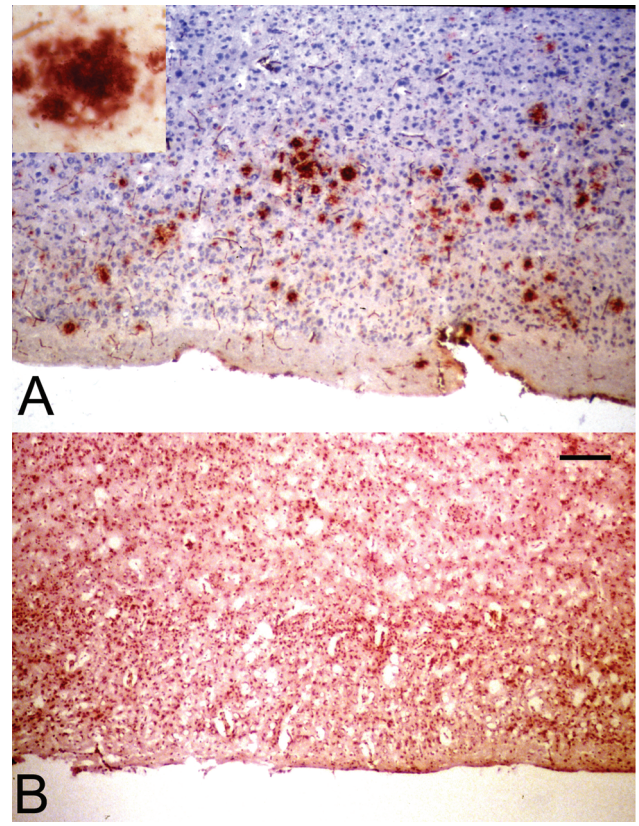

Figure 1. (A) Abeta immunocytochemistry of brain from a mouse in the saline treated group shows extensive immunoreactive Abeta amyloid plaques in the cortex. Inset: higher magnification of an amyloid plaque. (B) Prussian blue staining of brain from a mouse in the cTfRMAb-ScFv fusion protein treatment group shows no cerebral microhemorrhage. Magnification bar = 250 μm.

Table 1. Brain and Plasma Aβ^{1-42} Concentrations in AD Transgenic Mice[a]

treatment group	brain Aβ^{1-42} (ng/mg$_p$)	plasma Aβ^{1-42} (pg/mL)
saline	125 ± 14	<150
cTfRMAb-ScFv	75 ± 8*	<150

[a] Mean ± SE (n = 4 mice/group). *$p < 0.02$.

Discussion

This study shows that it is possible to decrease the Aβ^{1-42} amyloid burden in brain of AD transgenic mice with systemic AAA therapy without causing elevations in plasma Aβ^{1-42} peptide (Table 1) or cerebral microhemorrhage (Figure 1B). The lack of increase in plasma Aβ^{1-42} with chronic treatment with the cTfRMAb-ScFv fusion protein is due to the rapid removal of the fusion protein from the plasma compartment following iv injection, which is cleared with a mean residence time of less than 3 h in the mouse.[18] The cTfRMAb-ScFv fusion protein binds the TfR in peripheral tissues, which accelerates the removal from blood.[17] In contrast, with conventional AAA therapy, the AAA has a very long plasma

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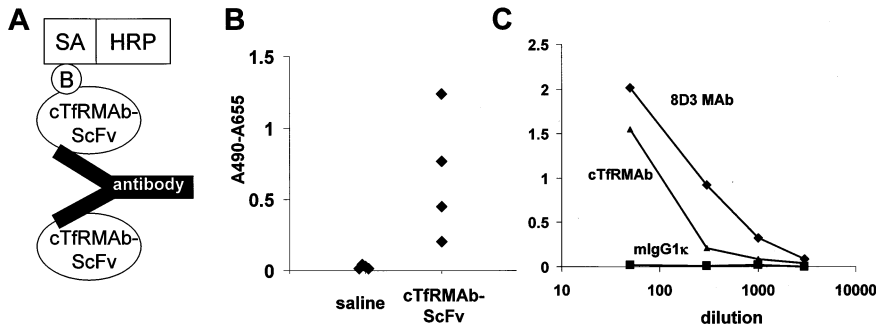

Figure 2. (A) Structure of the 2-site ELISA for detection of antibodies against the cTfRMAb-ScFv fusion protein. The cTfRMAb-ScFv fusion protein is used as the capture reagent, and the biotinylated cTfRMAb-ScFv fusion protein is used as the detector reagent, along with a complex of streptavidin (SA) and horseradish peroxidase (HRP); the biotin moiety is designated "B". (B) Absorbance at 1:50 dilutions of AD mouse plasma taken at the end of the 12-week treatment study for mice in either the saline or the cTfRMAb-ScFv fusion protein treatment groups. The capture reagent in this experiment is the cTfRMAb-ScFv fusion protein. (C) Absorbance at 1:50, 1:300, 1:1000, and 1:3000 dilutions of a pool of terminal plasma from the 2 mice in the cTfRMAb-ScFv fusion protein treatment group that reacted the highest in the screen at 1:50 dilution (panel B). The capture reagent in this experiment is the cTfRMAb antibody, the rat 8D3 antibody, or mouse IgG1κ.

residence time.[11] mAb drugs are large molecules, which exit the plasma compartment slowly with a half-time of days to weeks.[22] The typical AAA dose in treatment of AD mice is 10 mg/kg.[14] Following distribution of the AAA into the blood volume of a mouse, which is about 2 mL, the expected plasma concentration of the AAA would be about 1 μM. The circulating AAA causes Abeta peptide to redistribute to the plasma compartment, and the plasma Abeta peptide concentration after dosing with an AAA is 800 nM.[14] The plasma Aβ^{1-42} concentration in AD is normally very low, 40 pg/mL (0.01 nM).[23]

A side effect of conventional AAA therapy is brain microhemorrhage in the AD mouse.[13,14] The cause of cerebral microhemorrhage following AAA therapy in AD transgenic mice is not known, but it may be related to the elevation in plasma Abeta peptide that is associated with conventional AAA therapy. The administration of Aβ^{1-40} daily by iv injection causes cerebral microhemorrhage,[24] and the acute intracarotid arterial infusion of Aβ^{1-42} leads to a dose-dependent disruption of BBB permeability.[25] The BBB disruption that is concomitant with cerebral microhemorrhage may enable blood-borne AAAs, which normally do not cross the BBB, to penetrate the brain and access the amyloid plaque behind the BBB.[12]

The penetration of the BBB by an AAA therapeutic in AD in the absence of BBB disruption is possible following the re-engineering of the AAA as a fusion protein with a BBB molecular Trojan horse, such as the cTfRMAb. When the Trojan horse and the therapeutic are both antibodies, the problem becomes one of engineering a fusion protein from 2 separate antibodies. This was enabled by first re-engineering the AAA as a ScFv, followed by fusion of the amino terminus of the ScFv to the carboxyl terminus of the heavy chain of the BBB targeting antibody.[11] For delivery across the BBB in humans or Rhesus monkeys, the HIRMAb is used.[11,26] For delivery across the BBB in the mouse, the cTfRMAb is used.[18] Fusion of the AAA to the cTfRMAb has 2 advantages. First, the cTfRMAb targets the BBB TfR to enable penetration of the fused AAA into brain, without BBB disruption.[17,18] Second, the cTfRMAb targets the TfR in peripheral tissues, such as liver or spleen, to mediate the rapid removal of the AAA from the blood compartment.[17,18] Rapid clearance of the AAA from blood prevents the accumulation of Abeta amyloid peptide in the plasma compartment.

The chronic administration of the cTfRMAb-ScFv fusion protein could lead to immune reactions against the fusion protein. However, the cTfRMAb-ScFv fusion protein is a protein entirely of mouse amino acid sequence, with the exception of the variable regions of the cTfRMAb.[18] The variable regions, which originated from the rat 8D3 mAb against the mouse TfR,[17] appear to be

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the principal epitope on the cTfRMAb-ScFv fusion protein that is immunogenic. When the 8D3 mAb or the cTfRMAb is used as the capture reagent in the immunity ELISA, there is a significant immune signal (Figure 2C). However, there is no immune response directed against the constant region of the cTfMAb, as there is no immune signal recorded in the immunity ELISA when mouse IgG1κ is used as the capture reagent (Figure 2C). The immune response directed at the cTfRMAb-ScFv fusion protein is relatively low titer and seen at low dilutions of the terminal mouse plasma, such as 1:50 dilutions (Figure 2B). No clinically significant immune reactions were observed in any of the mice during the course of this 12 week study (Results). The immune tolerance is expected for an IgG therapeutic. The constant region of IgG expresses amino acid sequences, called Tregitopes, which induce immune tolerance.[27]

In summary, this pilot study in 12 month old AD transgenic mice demonstrates it is possible to reduce the Aβ amyloid burden in brain with systemic AAA therapy, and without the induction of either elevations in plasma Aβ peptide or cerebral microhemorrhage. However, this requires the re-engineering of the AAA as a fusion protein with a BBB molecular Trojan horse. In future studies, the goal is to achieve greater decreases in the brain amyloid burden by increasing either the duration of therapy beyond 12 weeks or the dose of the cTfRMAb-ScFv fusion protein. In addition, tumor necrosis factor (TNF)-α plays a role in Abeta amyloid accumulation in AD.[28,29] Therefore, biologic TNFα inhibitors (TNFI) may be therapeutic in AD. The type II TNF decoy receptor has been re-engineered for receptor-mediated delivery across the BBB.[30,31] A brain-penetrating biologic TNFI, used in conjunction with a brain-penetrating AAA, provides a new approach to AD drug development.

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