Published in final edited form as:

Bioconjug Chem. 2007 ; 18(2): 447–455. doi:10.1021/bc060349x.

Fusion Antibody for Alzheimer's Disease with Bi-Directional Transport Across the Blood-Brain Barrier and Abeta Fibril Disaggregation

Ruben J. Boado[†], Yufeng Zhang[†], Yun Zhang[†], Chun-Fang Xia[‡], and William M. Pardridge[‡]

[†] ArmaGen Technologies, Inc., 914 Colorado Ave., Santa Monica, CA

[‡] Department of Medicine, UCLA, Los Angeles, CA

Abstract

Delivery of monoclonal antibody therapeutics across the blood-brain barrier is an obstacle to the diagnosis or therapy of CNS disease with antibody drugs. The immune therapy of Alzheimer's disease attempts to disaggregate the amyloid plaque of Alzheimer's disease with an anti-Abeta monoclonal antibody. The present work is based on a 3-step model of immune therapy of Alzheimer's disease: (1) influx of the anti-Abeta monoclonal antibody across the blood-brain barrier in the blood to brain direction, (2) binding and disaggregation of Abeta fibrils in brain, and (3) efflux of the anti-Abeta monoclonal antibody across the blood-brain barrier in the brain to blood direction. This is accomplished with the genetic engineering of a tri-functional fusion antibody that binds (1) the human insulin receptor, which mediates the influx from blood to brain across the blood-brain barrier, (2) the Abeta fibril to disaggregate amyloid plaque, and (3) the Fc receptor, which mediates the efflux from brain to blood across the blood-brain barrier. This fusion protein is a new antibody-based therapeutic for Alzheimer's disease that is specifically engineered to cross the human blood-brain barrier in both directions.

Introduction

Monoclonal antibodies (MAb) have potential to be new pharmaceutical agents for the diagnosis or therapy of brain disease. However, MAb's are large molecule drugs that do not cross the blood-brain barrier (BBB). The immune therapy of Alzheimer's disease (AD) began with the active immunization of mice against the 40 amino acid amyloid Abeta (A β) peptide of AD (1). It was found that certain anti-Abeta antibodies could clear the brain of amyloid plaque following active immunization (1), which correlated with in vitro studies showing that certain anti-Abeta antibodies disaggregated pre-formed Abeta amyloid fibrils (2,3). The intra-cerebral injection of anti-Abeta antibodies in AD transgenic mice results in the rapid clearance of pre-existing plaque (4–6), and repair of dystrophic neurites (4,6). However, if anti-Abeta antibodies in the blood are to disaggregate the Abeta amyloid in brain, there must be a mechanism for circulating antibodies to cross the BBB, and enter brain from blood. In active immunization, the mice were immunized with Complete Freund's adjuvant (1), which enabled circulating anti-Abeta antibodies to enter brain from blood, because Complete Freund's adjuvant, and anti-mannan antibodies, cause BBB disruption (7,8). BBB disruption causes neuropathologic changes in the brain microvasculature (9) and in the brain (10). What is needed is an anti-Abeta MAb that is engineered to cross the BBB without the requirement for BBB disruption.

Large molecule drugs, such as antibody therapeutics, can cross the BBB, if the molecule is able to access specific receptor-mediated transport (RMT) systems within the BBB, such as the BBB insulin receptor or BBB transferrin receptor (11). A protein drug that is not a ligand for a BBB RMT system can still undergo transport across the BBB, if the MAb is conjugated to a BBB molecular Trojan horse. The latter is an endogenous ligand, or peptidomimetic MAb, that crosses the BBB via the endogenous RMT systems. Moreover, for certain brain diseases such as AD, there must also be a mechanism for efflux from brain back to blood of the complex of the therapeutic antibody and the Abeta peptide. Otherwise, there would be no net clearance of the Abeta amyloid peptide from AD brain. Therefore, an antibody therapeutic for AD must be engineered to enable transport across the BBB in both directions.

The present work describes the genetic engineering, expression, and validation of an anti-Abeta fusion antibody that is engineered to cross the BBB in both the blood to brain and the brain to blood directions. The immune therapy of AD is viewed as a 3-step process (Figure 1): (a) influx of the anti-Abeta antibody from blood to brain across the BBB, (b) binding to and disaggregation of Abeta fibrils behind the BBB, and (c) efflux of the Abeta-antibody complex from brain back to blood. The present studies describe the genetic engineering of a fusion antibody that is a tri-functional molecule. As shown in Figure 2, the “head” of the fusion antibody binds the human insulin receptor (HIR). The insulin receptor is highly expressed at the human BBB (12), and mediates the brain uptake of circulating insulin (13). In addition, the BBB insulin receptor mediates the brain uptake of certain peptidomimetic monoclonal antibodies (MAb) to the insulin receptor (14). The genetic engineering of either a chimeric or a humanized MAb to the HIR has been described previously (15,16), and both HIRMABs are rapidly transported from blood to brain across the BBB of the Rhesus monkey in vivo. The “tail” of the fusion antibody (Figure 2) is comprised of a single chain Fv (ScFv) antibody directed against the amino terminal portion of the Abeta peptide. The bivalent nature of the anti-Abeta MAb is restored when each ScFv monomer is fused to the carboxyl terminus of the heavy chain of the HIRMAB, as depicted in Figure 2. The “mid-section” of the fusion antibody contains the CH2-CH3 interface of the human IgG constant region, which is the binding site for the neonatal Fc receptor or FcRn (17). Unlike plasma proteins such as albumin, IgG molecules are rapidly exported unidirectionally from brain to blood across the BBB via a FcR (18). Confocal microscopy studies shows the principle BBB FcR isoform is the FcRn (19). The FcRn is an Fc receptor that is distinct from classical Fc receptors, and is responsible for transcytosis of IgG molecules in peripheral tissues (20). Following administration of the fusion antibody into the blood stream, the protein is enabled to cross the BBB in the blood to brain direction, bind and disaggregate Abeta fibrils in brain of AD, and undergo efflux from brain back to blood (Figure 1).

Experimental Procedures

Genetic engineering

The variable region of the heavy chain (VH) and the variable region of the light chain (VL) of the murine anti-A β ScFv were cloned by PCR using a cDNA template derived from VH and VL specific primers described previously (21), and poly A⁺ RNA isolated from the murine hybridoma cell line. Following PCR and agarose gel electrophoresis, the expected major cDNA bands corresponding to the PCR amplified ~0.4 kilobase (kb) VL cDNA and ~0.4 kb VH cDNA were obtained. The VH and VL bands were isolated from the agarose gels and subcloned into a ScFv vector to link the VH and VL genes aside a gene encoding a 17 amino acid linker; the VL gene was followed by sequence that encoded the 10 amino acid c-myc epitope (EQKLISEEDL) of the 9E10 MAb, as described previously (21). The anti-A β ScFv cDNA was subcloned into the pcDNA3.1 vector to produce the pCD-ScFv plasmid.

The engineering of the gene encoding the heavy chain of the fusion antibody was done in 2 steps: (a) PCR cloning of the anti-A β ScFv and (b) insertion of this cDNA into the universal chimeric HIRMAb heavy chain expression vector, pCD-UHC, to form the pCD-HC-ScFv plasmid. The pCD-UHC expresses the cDNA encoding for the intronless heavy chain (HC) of the chimeric HIRMAb (16), and has a single HpaI site at the end of the HIRMAb HC open reading frame (orf) for insertion of genes of interest and expression of HIRMAb HC fusion proteins. For the PCR cloning of the mature anti-A β ScFv, the mature ScFv PCR forward oligodeoxynucleotide (ODN) was designed, so as to delete the human IgG peptide leader sequence from the anti-A β ScFv cDNA, while maintaining the orf at the CH3 region of the HIRMAb heavy chain of the pCD-UHC. This results in the insertion of a Ser-Ser linker between the end of the CH3 region of the HIRMAb and the amino terminal part of the ScFv. The anti-A β ScFv reverse PCR ODN was designed to delete the c-myc tag, and to introduce a stop codon, TAA. Both PCR primers were 5'-phosphorylated for direct ligation into the pCD-UHC at the HpaI site. The PCR cloning of the mature anti-A β ScFv cDNA was done using the pCD-ScFv DNA as template. Agarose gel electrophoresis of the PCR products showed the expected single band of ~0.8 kb corresponding to the mature anti-A β ScFv cDNA. The engineered ScFv was ligated at the HpaI site in pCD-UHC to form the pCD-HC-ScFv expression vector. The pCD-HC-ScFv clone was validated by DNA sequencing. The deduced amino acid sequence showed the HC of the fusion antibody was comprised of 708 amino acids, and included a 18 amino acid signal peptide, a 113 amino acid HIRMAb VH, a 330 amino acid human IgG1 constant region, a 2-amino acid linker, a 114 amino acid anti-A β ScFv VH, a 17 amino acid linker, and a 113 amino acid anti-A β ScFv VL. The predicted molecular weight of the fusion antibody heavy chain, minus the signal peptide, was 75,594 Da, and the predicted isoelectric point (pI) was 8.81. This pI was experimentally confirmed by isoelectric focusing.

Binding of anti-A β ScFv to A β ¹⁻⁴⁰ and A β plaque

COS cells were transfected with the pCD-ScFv plasmid with Lipofectamine 2000, and conditioned medium was tested in an ELISA. Soluble A β ¹⁻⁴⁰ peptide was adsorbed to 96-well plates, and COS cell conditioned medium was added. The primary antibody was a murine 9E10 to the c-myc epitope, and 9E10 binding to the ScFv was detected with an anti-mouse IgG-peroxidase conjugate. This format was also used in Western blotting. Concentrated COS cell medium was applied to a 12% SDS-PAGE gel, and the proteins were electroblotted to nitrocellulose. The primary antibody was the 9E10 antibody, and the secondary antibody was an anti-mouse IgG-peroxidase conjugate. Frozen sections (20 μ m) of AD brain were incubated with COS cell medium, and ScFv binding to plaque was detected with the 9E10 antibody.

Expression of intact fusion antibody in COS cells

COS cells were dual transfected with pCD-HC-ScFv and pCD-LC, which is the chimeric HIRMAb LC expression plasmid (16), and conditioned medium was collected at 3 and 6 days. The fusion antibody was purified by protein A affinity chromatography, and the purity was assessed with both 12% SDS-PAGE followed by Coomassie blue staining, and by Western blotting using an anti-human IgG primary antibody.

Binding of fusion antibody to A β and to HIR

The binding of the fusion antibody to soluble A β ¹⁻⁴⁰ was measured with an immunoradiometric assay described previously (22). The A β ¹⁻⁴⁰ is plated in a 96-well format, and the binding of [¹²⁵I]-murine anti-A β MAb to the solid phase A β ¹⁻⁴⁰ is quantitated. The percent displacement of this binding is measured in the presence of increasing concentrations of either unlabeled murine anti-A β MAb or the fusion antibody. Binding to the HIR extracellular domain (ECD) was measured as described previously (15). HIR ECD was affinity purified from conditioned medium obtained from CHO cells permanently transfected with the HIR ECD. The HIR ECD

was plated in a 96-well format, and binding of the HIRMAb was measured with an anti-human IgG primary antibody and a peroxidase-based ELISA. The binding of the fusion antibody or human IgG1 to the HIR ECD was measured in parallel. Binding isotherms were generated by non-linear regression analysis using the PAR subroutine of the BMDP Statistical Software.

Blood to brain transport in the primate in vivo

The fusion antibody was iodinated with [125 I]-iodine and chloramine T. In parallel, the murine anti-A β MAb was tritiated with [3 H]-N-succinimidyl propionate. A 8 year old female Rhesus monkey, weighing 10.2 kg, was administered by a single intravenous injection a dose of 777 μ Ci of [125 I]-fusion antibody and 888 uCi of [3 H]-murine anti-A β MAb. Serum was collected at multiple time points over a 180 min period. The serum removed from the anesthetized Rhesus monkey was analyzed for total radioactivity, and expressed as a % of injected dose (I.D.)/mL serum. The 125 I radioactivity was counted in a gamma counter, and the 3 H radioactivity was counted in a liquid scintillation counter; the 125 I isotope emits radioactivity in the 3 H window and standard curves were prepared to account for 125 I emission in the 3 H channel. The serum concentration profile for the [125 I]-fusion antibody was fit to a 2-compartment pharmacokinetic model described previously (14) to yield the pharmacokinetic parameters. At 180 minutes after drug injection, the animal was euthanized, and brain radioactivity was analyzed with the capillary depletion method, as described previously (23). This method separates brain homogenate into a capillary pellet and a post-vascular supernatant. If the volume of distribution (VD) of the antibody in the post-vascular supernatant is high, then this is evidence that the antibody has crossed the BBB and entered into the brain interstitial and intracellular spaces. The VD has units of uL/gram brain and is the ratio of the concentration of the antibody in brain (DPM/g) divided by the concentration of the antibody in serum (DPM/uL) at the 180 terminal time point. The primate brain was divided into frontal, mid-section, and caudal parts, and coronal slabs were snap frozen in pulverized dry ice, and 20 μ m frozen sections were prepared on a cryostat. The sections were exposed to Biomax x-ray film for 7–10 days, and the film was scanned, and the grayscale image was colorized with NIH Image software.

Brain to blood transport in the rat in vivo

Human IgG is recognized by the rat BBB Fc receptor (FcR) (18), and by the rodent FcRn (24). The [125 I]-fusion antibody (0.03 uCi in 0.3 uL) was injected into the cortex of the brain of the anesthetized adult rat under stereotaxic guidance per the standard protocol of the Brain Efflux Index (BEI) technique (18). The fusion antibody was injected in the par2 region of the parietal cortex of brain, with the following stereotaxic coordinates: 0.2 mm anterior to bregma; 5.5 mm lateral to bregma; 4.5 mm deep from the dural surface. This region is far removed from the cerebrospinal fluid tracts, and efflux of radioactivity from brain over time can only occur via efflux across the BBB from brain to blood. The labeled fusion antibody was injected with either 0 or 1.5 μ g human Fc fragments, as described previously (18). The rate of efflux of the [125 I]-fusion antibody from rat brain was followed over 90 minutes.

Binding and in vitro disaggregation of A β fibrils by fusion antibody

A β fibrils were formed after incubating A β^{1-40} peptide (Bachem) in an orbital shaker at 37C for 6 days (25), and the plaque was collected by centrifugation. An affinity purified rabbit polyclonal antibody against the carboxyl terminus (CT) of the A β^{1-40} peptide (Signet Laboratories, catalogue number 9130-005) was plated in 96-well dishes. In parallel, the pre-formed A β fibrils were incubated for either 1 or 4 hours at 37C with either the fusion antibody, human IgG1 (hIgG1), phosphate buffered saline (PBS), or with the mouse anti-A β MAb, mouse IgG (mIgG), or PBS. The A β aggregate/fusion antibody, or A β aggregate/mouse anti-A β MAb complex was then added in increasing doses (10, 30, 100 uL, which is equivalent to 100, 300, 1000 ng/mL) to the immobilized anti-CT antibody. The anti-A β ScFv part of the fusion

antibody, or the mouse anti-A β MAb, binds an epitope on the A β ¹⁻⁴⁰ peptide near the amino terminus (NT). Therefore, if A β fibrils are present, then a complex will form between anti-CT antibody, the A β fibril, the fusion antibody, and a secondary antibody coupled to peroxidase for detection of anti-A β antibody binding to A β fibril by ELISA (2,3). The secondary antibodies used for the fusion antibody and the murine anti-A β MAb were anti-human and anti-mouse IgG conjugates of alkaline phosphatase, respectively.

In vivo disaggregation of A β plaque by fusion antibody in transgenic mice

APPswe/PSEN1(dE9) double transgenic (26) male mice were obtained from The Jackson Laboratory (Bar Harbor, ME), and investigated at 15 months of age. Following approval by the UCLA Animal Research Committee, mice were anesthetized with ketamine/xylazine and the fusion antibody (20 pmol in 2 μ L), or saline (2 μ L) was injected with a mouse stereotaxis apparatus into the cortex and hippocampus on the right side of the brain with the following coordinates: 1.5 mm anterior, 2.0 mm lateral, and 2.0 mm deep relative to bregma and the dural surface for the frontal cortex; and 2.7 mm posterior, 2.5 mm lateral, and 2.5 mm deep relative to bregma and the dural surface for the hippocampus. The mice were euthanized 48 hours later, and the brain was removed and coronal slabs around the cortex and hippocampal injection sites were immersion fixed 24 hours at 4C in 4% paraformaldehyde in PBS, and cryo-protected successively in 10%, 20%, and 30% sucrose. The cortex or the hippocampus slab was frozen in embedding medium and 40 consecutive 20 micron frozen sections were prepared on a Mikron cryostat at -15C. The slides were stained with either 4% thioflavine-S for confocal microscopy or 1% potassium ferrocyanide in 1% HCl for Prussian blue light microscopy. For examination of micro-hemorrhage, every 8th section was stained for Prussian blue deposits. For quantitation of amyloid plaque, every 5th section was examined by confocal microscopy using a Zeiss confocal microscope and Zeiss LSM software (LSM 5 PASCAL, version 3.2) for digitizing the area of amyloid plaque, relative to the field area, which was 52,642 μ m². For each of the 8 sections, 8 separate fields were quantified and averaged for both the injected side and the non-injected side of brain. The difference in amyloid content in the injected brain vs the contralateral, non-injected brain, for either the animals injected with fusion antibody, or with saline, was assessed with Student's t-test.

Results

The cDNAs encoding the variable region of the heavy chain (VH) and variable region of the light chain (VL) were cloned following reverse transcription of RNA isolated from a mouse hybridoma secreting a MAb directed against the A β ¹⁻²⁸ peptide. The VH and VL were joined by a 17 amino acid linker to produce a ScFv. The 10-amino acid c-myc epitope of the 9E10 MAb was fused to the carboxyl terminus of the ScFv, followed by insertion in a eukaryotic expression plasmid, designated pCD-ScFv (Methods). Following transfection of COS cells, the reactivity of the engineered anti-A β ScFv against the A β peptide was tested with 3 assays: Western blot, ELISA, and immunocytochemistry of AD brain. Western blotting with the 9E10 antibody showed that a 31 kDa immunoreactive protein was selectively secreted to the medium in COS cells transfected with pCD-ScFv. The anti-A β ScFv secreted by transfected COS cells bound soluble A β ¹⁻⁴⁰ as demonstrated with a 9E10 antibody-based ELISA (Methods). Immunocytochemistry of frozen sections of AD autopsy brain showed the anti-A β ScFv specifically bound to vascular amyloid plaque (Figures 3a and c), whereas medium conditioned by COS cells exposed only to lipofectamine 2000 (Figure 3b), or the mouse IgG isotype control antibody (Figure 3d), showed no specific staining of AD brain.

The cDNA encoding the anti-A β ScFv was fused at the 3' end of the cDNA encoding the heavy chain (HC) of the genetically engineered HIRMAb and this heavy chain fusion antibody expression vector is designated pCD-HC-ScFv. A separate expression vector, designated pCD-

LC, encodes the light chain of the HIRMAb (15,16). COS cells were dual transfected with pCD-HC-ScFv and pCD-LC, and the fusion antibody secreted into the medium was purified by protein A affinity chromatography. Following SDS-PAGE and Coomassie blue staining, the size of the HC of the fusion antibody was 82 kDa, whereas the size of the HC of HIRMAb was 55 kDa; the difference, 27 kDa, is equal to the size of the ScFv, minus the 9E10 epitope. The size of the light chain (LC) of either the HIRMAb or the fusion antibody is identical at 28 kDa (Figure 4a). The same sizes of the HC and LC of the fusion antibody and the HIRMAb were detected with a Western blot using a primary antibody against human IgG (Figure 4b).

The fusion antibody bound with high affinity to soluble A β ¹⁻⁴⁰, as determined by an immunoradiometric assay (Methods). The dissociation constant (K_D) of the original murine anti-Abeta tetrameric MAb was 32 $\pm$ 11 nM, and the dissociation constant of fusion antibody binding to soluble A β ¹⁻⁴⁰ was 24 $\pm$ 4 nM (Figure 5a). The fusion antibody retained high affinity binding for the HIR based on an ELISA (Methods) using affinity purified HIR extracellular domain (ECD). The concentration of HIRMAb that gives 50% binding to HIR ECD is 0.53 $\pm$ 0.02 nM, of fusion antibody binding to the HIR ECD is 1.0 $\pm$ 0.1 nM (Figure 5b).

The fusion antibody was radiolabeled with 125-iodine, the hybridoma-derived mouse anti-Abeta MAb was tritiated, and the labeled antibodies were co-injected intravenously into an anesthetized adult female Rhesus monkey. In the first 3 hours after injection, there was no measurable decrease in the blood concentration of the [³H]-mouse Abeta MAb, whereas there was an immediate clearance of the [¹²⁵I]-fusion antibody from blood in the primate (Figure 6a). The extensive distribution of the fusion antibody into the primate brain is shown by the 3 hour brain scan (Figure 7). There is global uptake of the fusion antibody in all parts of the brain with a preferential uptake in gray matter relative to white matter. Brain radioactivity at 3 hours after intravenous injection was measured with the capillary depletion technique, which demonstrated a high volume distribution (VD), >100 uL/g, of the [¹²⁵I]-fusion antibody (Figure 6b). In contrast, the brain VD for the [³H]-mouse anti-Abeta MAb is 10 μ L/g brain. This VD is equal to the arterial blood volume of brain (27), and indicates the mouse anti-Abeta MAb does not cross the BBB in the blood to brain direction. The VD of the fusion antibody was high in the postvascular supernatant, indicating the fusion antibody is transcytosed across the BBB into the postcapillary compartment of brain (Figure 6b).

The influx of the fusion antibody from blood to brain in vivo could not be tested in rodents, because the HIRMAb does not recognize the rodent insulin receptor. However, the rat FcRn does recognize human IgG, and mediates efflux of human IgG across the BBB from brain to blood in the rat (18). Therefore, the transport of the fusion antibody across the BBB in the brain to blood direction was measured with the Brain Efflux Index (BEI) method in the adult rat (Methods). Following stereotaxic injection into the cortex of rat brain, the fusion antibody rapidly effluxed from brain to blood, and was >50% eliminated from brain at 90 minutes after injection. The efflux of the fusion antibody from brain was >90% blocked by human Fc fragments (Figure 8).

The binding of the fusion antibody to Abeta fibrils in vitro is demonstrated with the double antibody ELISA outlined in Figure 9a. The fusion antibody bound to the Abeta fibrils in a dose dependent manner following a 1 hour incubation of the fusion antibody with the Abeta fibrils (Figure 9b). Following a 4 hour incubation of the fusion antibody and the Abeta fibrils, most of the fibrils were disaggregated (Figure 9b). A comparable binding/disaggregation of Abeta fibrils was observed with the original murine anti-Abeta MAb. The fusion antibody also disaggregates Abeta amyloid in vivo in transgenic mouse brain. The APPswe/PS1dE9 double transgenic mouse develops amyloid plaque as shown by thioflavin-S staining (Figure 9c). Quantitation of the percent of either frontal cortex or hippocampus occupied by the amyloid plaque showed that approximately 5% of the brain was amyloid. A single intra-cerebral

injection of the fusion antibody into the hippocampus or the frontal cortex caused a $39 \pm 3\%$ and $38 \pm 2\%$ decrease, respectively, in the Abeta amyloid burden in brain in vivo at 48 hours after injection in the side ipsilateral to the injection, relative to the amyloid burden in the contralateral hippocampus or frontal cortex (Figure 9d). Conversely, the intra-cerebral injection of saline in either the cortex or hippocampus of the transgenic mice caused no decrease in amyloid burden in the brain (Figure 9d). Intra-cerebral injection of the fusion antibody caused no micro-hemorrhage in brain, as demonstrated by Prussian blue staining of serial sections of cortex or hippocampus (Methods).

Discussion

The results of these studies are consistent with the following conclusions. First, a ScFv directed against the amino terminal portion of the A β peptide has been cloned, expressed, and shown to both bind soluble Abeta and Abeta fibrils (Results and Figure 3). Second, a fusion antibody has been engineered whereby the anti-Abeta ScFv is fused to the carboxyl terminus of the Fc region of a genetically engineered HIRMAb (Figures 2 and 4); the fusion antibody retains high affinity binding for both the HIR (Figure 5b), as well as for soluble Abeta (Figure 5a) and for Abeta fibrils (Figure 9b). Third, the fusion antibody crosses the BBB bidirectionally; the fusion antibody undergoes rapid influx from blood to brain across the Rhesus monkey BBB in vivo (Figure 7); the fusion antibody undergoes receptor-mediated efflux from brain to blood across the BBB via a mechanism that is inhibited by Fc fragments (Figure 8). Fourth, the fusion antibody (Figure 2) is a tri-functional protein with three domains that are all biologically active. The first domain, which targets the HIR, enables receptor-mediated transport across the BBB in the blood to brain direction. The second domain binds to Abeta to cause disaggregation of Abeta fibrils. The third domain binds the Fc receptor to enable receptor-mediated transport of the fusion antibody from brain back to blood across the BBB.

The availability of the cDNA encoding the anti-Abeta ScFv and the cDNA encoding the heavy chain of the chimeric HIRMAb enabled the genetic engineering of a fusion antibody with dual specificities. The fusion antibody binds both the HIR and Abeta (Figure 2). There are multiple approaches to the genetic engineering of fusion antibodies (28–31). The approach taken in the present investigation was to fuse the ScFv at the carboxyl terminus of the CH3 region of the HIRMAb heavy chain (Figure 2). Prior work has shown that the fusion of a ScFv to the carboxyl terminus of the CH3 region of another IgG results in an approximate 10-fold reduction in binding affinity for the ScFv-targeted antigen (28,32). This was not observed in the present investigation, as the affinity of the fusion antibody for Abeta is unchanged as compared to the original murine anti-Abeta MAb (Figure 5a). The advantage of fusing the ScFv to the carboxyl terminus of the Fc region of the HIRMAb is that this configuration converts the monovalency of ScFv binding of antigen to the original bivalency of antibody binding of antigen (Figure 2).

COS cells were dual transfected with a heavy chain expression vector, pCD-HC-ScFv, and an expression vector, pCD-LC, which produces the light chain of the genetically engineered HIRMAb. Medium was concentrated and the fusion antibody was purified by protein A affinity chromatography. SDS-PAGE and Western blotting showed the fusion antibody was correctly processed in COS cells. The size of the light chain of the fusion antibody is identical to the size of the light chain of the HIRMAb (Figure 4), which is expected since both the fusion antibody and the HIRMAb are comprised of the same light chain. The size of the heavy chain of the fusion antibody, 82 kDa, is 27 kDa greater than the size of the heavy chain of the HIRMAb, which is 55 kDa (Figure 4). The difference between the size of the heavy chain of the fusion antibody and the size of the HIRMAb, 27 kDa, is equal to the predicted molecular weight of the ScFv. The bi-functionality of the fusion antibody was demonstrated by separate binding assays targeting either the HIR ECD or soluble A β^{1-40} . The fusion antibody binds soluble A β^{1-40} with a K_D of 24 ± 4 nM and this is not significantly different from the K_D , 32

± 11 nM, of the binding to $A\beta^{1-40}$ of the original murine anti-Abeta MAb (Figure 5a). The 50% binding of the HIRMAb to the HIR ECD is observed at 0.53 ± 0.02 nM and there is a $>50\%$ retention of affinity of the fusion antibody for the HIR ECD (Figure 5b). There is no binding of human IgG1 to the HIR (Figure 5b). The fusion antibody binds soluble $A\beta^{1-40}$ (Figure 5a), and also binds Abeta fibrils (Figure 9b). After a 4-hour incubation with Abeta fibrils, the fusion antibody causes a time-dependent disaggregation of Abeta fibrils (Figure 9b). The fusion antibody also causes disaggregation of amyloid plaque in vivo in double transgenic APPswe/PS1dE9 mice (Figure 9d). A single intra-cerebral injection of 20 pmol of fusion antibody into either the frontal cortex or the hippocampus causes a 38–39% reduction in brain amyloid burden at 48 hours after fusion antibody administration (Figure 9d). In contrast, the injection of saline into the frontal cortex or hippocampus caused no decrease in brain amyloid burden (Figure 9d). The mechanism of fusion antibody disaggregation of preformed Abeta plaque is assumed to involve binding of the antibody to amino terminal epitopes on the Abeta peptide within the fibril, which induces progressive release of Abeta monomers, and/or oligomers, from the fibril (2,3).

The purpose of this investigation was to genetically engineer an anti-Abeta antibody that can also cross the BBB in both the blood to brain and brain to blood directions. Therefore, an anti-Abeta ScFv antibody was fused to the heavy chain of the genetically engineered HIRMAb to enable receptor-mediated transport in the blood to brain direction across the BBB via transport on the endogenous insulin receptor. The HIRMAb is not recognized by the rodent insulin receptor, but is recognized by the insulin receptor of Old World primates such as Rhesus monkeys, but not New World primates such as squirrel monkeys (14). The selective transport of the fusion antibody across the primate BBB, relative to the murine anti-Abeta MAb is demonstrated in Figure 6b. There is no measurable clearance of the murine anti-Abeta MAb from primate blood at 3 hours after intravenous administration (Figure 6a), because this antibody, which is a mouse IgG1, does not recognize any receptor that would mediate selective exodus from the blood compartment. The human FcRn, and presumably the primate FcRn, does not recognize mouse IgG1 (24). However, the lack of recognition of the murine anti-Abeta MAb by the primate FcRn cannot explain the lack of influx of this MAb from blood to brain. The BBB FcRn is an asymmetric transporter, and mediates only the efflux of IgG from brain to blood, but not the influx of IgG from blood to brain (18). In contrast to the slow clearance of the murine anti-Abeta MAb from blood, the fusion antibody is rapidly removed from the blood (Figure 6a), owing to uptake via tissues such as liver, spleen, and brain that express vascular insulin receptor (14). This rapid removal of fusion antibody is likely mediated by the insulin receptor, since the rate of removal of the fusion antibody from the blood compartment of the Rhesus monkey is virtually identical to the rate of removal of the genetically engineered HIRMAb reported previously (15,16). The systemic clearance of the [^{111}In]-chimeric HIRMAb, 0.22 ± 0.08 ml/min/kg (15), is not significantly different from the systemic clearance of the [^{125}I]-fusion antibody, 0.18 ± 0.05 ml/min/kg. The 3-hour brain scan of the primate shows global distribution of the fusion antibody in the primate brain in vivo with a greater uptake in gray matter relative to white matter (Figure 7). This pattern of uptake reflects the approximate 3-fold greater vascular density in gray matter relative to white matter, and thus 3-fold greater density of microvascular insulin receptor in gray matter as compared to white matter (14). The rapid transport of fusion antibody across the primate BBB in vivo is confirmed by measurements of brain VD (Figure 6b). The brain VD of the murine anti-Abeta MAb, 10 $\mu\text{L/g}$ (Figure 6b), is equal to the cerebral arterial blood volume (27), which indicates the murine anti-Abeta MAb does not cross the BBB in vivo in the blood to brain direction. In contrast, the brain VD for the fusion antibody is >100 $\mu\text{L/g}$ brain (Figure 6b). Capillary depletion analysis shows that the fusion antibody undergoes transcytosis across the BBB and penetrates into the postvascular volume of brain (Figure 6b). Once inside brain, an anti-Abeta antibody may bind amyloid and cause disaggregation of the amyloid plaque.

In addition to influx from blood to brain, an Abeta antibody therapeutic must also be enabled to efflux from brain back to blood. Otherwise, there would be no net clearance of amyloid from brain. Large molecule efflux across the BBB may be quantitated with the Brain Efflux Index method (18). Prior work shows that during a 90 min period after intra-cerebral injection, the amount of 70 kDa [³H]-dextran in rat brain is $93 \pm 3\%$, which means that the efflux from brain via bulk flow is minimal during this time period. Therefore, the only pathway for efflux from brain is direct transport across the BBB. Unlike other large molecules such as dextran or albumin, IgG molecules rapid efflux from brain to blood via a BBB Fc receptor (18). Previous confocal microscopy studies have shown that the neonatal FcR, also called the FcRn, is highly expressed at the brain microvascular endothelium (19). The FcRn binding site for IgG molecules is at the CH₂-CH₃ interface (17), and the FcRn binds deglycosylated IgG (33). Therefore, the fusion antibody was engineered to have an intact CH₂-CH₃ to enable binding and transport via the BBB FcRn (Figure 2). After the complex of A β and the fusion antibody efflux from brain to blood, the fusion antibody is rapidly cleared from blood by tissues such as liver or spleen, which express high levels of microvascular insulin receptor (14).

The potential immunogenicity of the fusion antibody in humans is not known until clinical trials are performed. The fusion antibody is derived from chimeric anti-HIR and anti-Abeta antibodies, and both chimeric monoclonal antibodies, and antibody fusion proteins, are FDA approved products, which are given repeatedly to humans without immunologic reactions (34). Any underlying immunogenicity of the fusion antibody in humans could be reduced by "humanization" of either or both parts of the fusion antibody. A humanized HIRMAb has been genetically engineered, and crosses the primate BBB at rates comparable to the chimeric HIRMAb (16).

The genetic engineering of the fusion antibody shown in Figure 2 could be replicated with other monoclonal antibody-based therapeutics for multiple CNS diseases. Monoclonal antibody based therapeutics have significant potential as neurotherapeutics, if the problem of BBB delivery is solved. The present work shows that antibody based therapeutics can be delivered to brain as fusion proteins with molecular Trojan horses that cross the BBB.

Acknowledgements

This work was supported by NIH grant R43-NS-51857.

References

1. Schenk D, Barbour R, Dunn W, Gordon G, Grajeda H, Guido T, Hu K, Huang J, Johnson-Wood K, Khan K, Kholodenko D, Lee M, Liao Z, Lieberburg I, Motter R, Mutter L, Soriano F, Shopp G, Vasquez N, Vandevert C, Walker S, Wogulis M, Yednock T, Games D, Seubert P. Immunization with amyloid-beta attenuates Alzheimer-disease-like pathology in the PDAPP mouse. *Nature* 1999;400:173–177. [PubMed: 10408445]
2. Solomon B, Koppel R, Frankel D, Hanan-Aharon E. Disaggregation of Alzheimer beta-amyloid by site-directed mAb. *Proc Natl Acad Sci USA* 1997;94:4109–4112. [PubMed: 9108113]
3. Solomon B, Koppel R, Hanan E, Katzav T. Monoclonal antibodies inhibit in vitro fibrillar aggregation of the Alzheimer beta-amyloid peptide. *Proc Natl Acad Sci USA* 1996;93:452–455. [PubMed: 8552659]
4. Lombardo JA, Stern EA, McLellan ME, Kajdasz ST, Hickey GA, Bacskai BJ, Hyman BT. Amyloid-beta antibody treatment leads to rapid normalization of plaque-induced neuritic alterations. *J Neurosci* 2003;23:10879–10883. [PubMed: 14645482]
5. Wilcock DM, DeCarlo G, Henderson D, Jackson J, Clarke K, Ugen KE, Gordon MN, Morgan D. Intracranially administered anti-A beta antibodies reduce beta-amyloid deposition by mechanisms both independent of and associated with microglial activation. *J Neurosci* 2003;23:3745–3751. [PubMed: 12736345]

6. Brendza RP, Bacsikai BJ, Cirrito JR, Simmons KA, Skoch JM, Klunk WE, Mathis CA, Bales KR, Paul SM, Hyman BT, Holtzman DM. Anti-Abeta antibody treatment promotes the rapid recovery of amyloid-associated neuritic dystrophy in PDAPP transgenic mice. *J Clin Invest* 2005;115:428–433. [PubMed: 15668737]
7. Rabchevsky AG, Degos JD, Dreyfus PA. Peripheral injections of Freund's adjuvant in mice provoke leakage of serum proteins through the blood-brain barrier without inducing reactive gliosis. *Brain Res* 1999;832:84–96. [PubMed: 10375654]
8. Namer IJ, Steibel J. Antibody directed against mannan of the *Mycobacterium tuberculosis* cell envelope provokes blood-brain barrier breakdown. *J Neuroimmunol* 2000;103:63–68. [PubMed: 10674990]
9. Lossinsky AS, Vorbrodt AW, Wisniewski HM. Scanning and transmission electron microscopic studies of microvascular pathology in the osmotically impaired blood-brain barrier. *J Neurocytol* 1995;24:795–806. [PubMed: 8586999]
10. Salahuddin TS, Johansson BB, Kalimo H, Olsson Y. Structural changes in the rat brain after carotid infusions of hyperosmolar solutions. An electron microscopic study. *Acta Neuropathol (Berl)* 1988;77:5–13. [PubMed: 3149121]
11. Pardridge WM. Development. Cambridge University Press; Cambridge, United Kingdom: 2001. *Brain Drug Targeting; The Future of Brain Drug*; p. 1-370.
12. Pardridge WM, Eisenberg J, Yang J. Human blood-brain barrier insulin receptor. *J Neurochem* 1985;44:1771–1778. [PubMed: 2859355]
13. Duffy KR, Pardridge WM. Blood-brain barrier transcytosis of insulin in developing rabbits. *Brain Res* 1987;420:32–38. [PubMed: 3315116]
14. Pardridge WM, Kang YS, Buciak JL, Yang J. Human insulin receptor monoclonal antibody undergoes high affinity binding to human brain capillaries in vitro and rapid transcytosis through the blood-brain barrier in vivo in the primate. *Pharm Res* 1995;12:807–816. [PubMed: 7667183]
15. Coloma MJ, Lee HJ, Kurihara A, Landaw EM, Boado RJ, Morrison SL, Pardridge WM. Transport across the primate blood-brain barrier of a genetically engineered chimeric monoclonal antibody to the human insulin receptor. *Pharm Res* 2000;17:266–274. [PubMed: 10801214]
16. Boado RJ, Zhang Y-F, Zhang Y, Pardridge WM. Humanization of anti-human insulin receptor antibody for drug targeting across the human blood-brain barrier. *Biotechnol Bioeng*. 2007In press
17. Martin WL, West AP Jr, Gan L, Bjorkman PJ. Crystal structure at 2.8 angstrom of an FcRn/heterodimeric Fc complex: mechanism of pH-dependent binding. *Mol Cell* 2001;7:867–877. [PubMed: 11336709]
18. Zhang Y, Pardridge WM. Mediated efflux of IgG molecules from brain to blood across the blood-brain barrier. *J Neuroimmunol* 2001;114:168–172. [PubMed: 11240028]
19. Schlachetzki F, Zhu C, Pardridge WM. Expression of the neonatal Fc receptor (FcRn) at the blood-brain barrier. *J Neurochem* 2002;81:203–206. [PubMed: 12067234]
20. Ghetie V, Ward ES. Multiple roles for the major histocompatibility complex class I-related receptor FcRn. *Annu Rev Immunol* 2000;18:739–766. [PubMed: 10837074]
21. Li JY, Sugimura K, Boado RJ, Lee HJ, Zhang C, Duebel S, Pardridge WM. Genetically engineered brain drug delivery vectors-cloning, expression, and in vivo application of an anti-transferrin receptor single chain antibody-streptavidin fusion gene and protein. *Protein Engineering* 1999;12:787–796. [PubMed: 10506289]
22. Pardridge WM, Bickel U, Buciak J, Yang J, Diagne A, Aepinus C. Cationization of a monoclonal antibody to the human immunodeficiency virus rev protein enhances cellular uptake but does not impair antigen binding of the antibody. *Immunol Lett* 1994;42:191–195. [PubMed: 7890319]
23. Triguero D, Buciak JB, Pardridge WM. Capillary depletion method for quantifying blood-brain barrier transcytosis of circulating peptides and plasma proteins. *J Neurochem* 1990;54:1882–1888. [PubMed: 2338547]
24. Ober RJ, Radu CG, Ghetie V, Ward ES. Differences in promiscuity for antibody-FcRn interactions across species: implications for therapeutic antibodies. *Int Immunol* 2001;13:1551–1559. [PubMed: 11717196]
25. Shearman MS, Hawtin SR, Tailor VJ. The intracellular component of cellular 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction is specifically inhibited by beta-amyloid peptides. *J Neurochem* 1995;65:218–227. [PubMed: 7790863]

26. Savonenko A, Xu GM, Melnikova T, Morton JL, Gonzales V, Wong MP, Price DL, Tang F, Markowska AL, Borchelt DR. Episodic-like memory deficits in the APPswe/PS1dE9 mouse model of Alzheimer's disease: Relationship to β -amyloid deposition and neurotransmitter abnormalities. *Neurobiol Dis* 2005;18:602–617. [PubMed: 15755686]
27. Ito H, Ibaraki M, Kanno I, Fukuda H, Miura S. Changes in the arterial fraction of human cerebral blood volume during hypercapnia and hypocapnia measured by positron emission tomography. *J Cereb Blood Flow Metab* 2005;25:852–857. [PubMed: 15716851]
28. Coloma MJ, Morrison SL. Design and production of novel tetravalent bispecific antibodies. *Nature* 1997;15:159–163.
29. Lu D, Jimenez X, Zhang H, Bohlen P, Witte L, Zhu Z. Fab-scFv fusion protein: an efficient approach to production of bispecific antibody fragments. *J Immunol Methods* 2002;267:213–226. [PubMed: 12165442]
30. Merchant AM, Zhu Z, Yuan JQ, Goddard A, Adams CW, Presta LG, Carter P. An efficient route to human bispecific IgG. *Nature* 2003;16:677–681.
31. Miller K, Meng G, Liu J, Hurst A, Hsei V, Wong WL, Ekert R, Lawrence D, Sherwood S, DeForge L, Gaudreault J, Keller G, Sliwkowski M, Ashkenazi A, Presta L. Design, construction, and in vitro analyses of multivalent antibodies. *J Immunol* 2003;170:4854–4861. [PubMed: 12728922]
32. Marvin JS, Zhu Z. Recombinant approaches to IgG-like bispecific antibodies. *Acta Pharmacol Sin* 2005;6:649–658. [PubMed: 15916729]
33. Shields RL, Lai J, Keck R, O'Connell LY, Hong K, Meng YG, Wikert SH, Presta LG. Lack of fucose on human IgG1 N-linked oligosaccharide improves binding to human Fc γ RIII and antibody-dependent cellular toxicity. *J Biol Chem* 2002;277:26733–26740. [PubMed: 11986321]
34. Waldmann TA, Morris JC. Development of antibodies and chimeric molecules for cancer immunotherapy. *Adv Immunol* 2006;90:83–131. [PubMed: 16730262]

Figure 1. Import-export model of Abeta antibody therapeutics

The fusion antibody clears amyloid from brain in AD via 3 sequential steps, and each of these 3 sequential steps uses separate parts of the fusion antibody molecule. Step 1 is the influx of the fusion antibody from blood to brain across the BBB, which is mediated by binding of the fusion antibody to the BBB HIR. Step 2 is binding of the fusion antibody to the amyloid plaque in AD, which promotes disaggregation of the amyloid plaque, and this binding to the plaque is mediated by the anti- $A\beta$ ScFv part of the fusion antibody. Step 3 is the efflux of the fusion antibody from brain to blood across the BBB, which is mediated by binding of the fusion antibody to the BBB FcRn at the CH2-CH3 interface of the Fc region of the fusion antibody.

Figure 2. Antibody fusion protein with 3 functional domains

The first domain, the HIRMAb, binds the BBB HIR to trigger influx across the BBB. The second domain, the CH2/CH3 interface of the Fc region, binds to the BBB FcRn to trigger efflux from brain back to blood. The third domain, the anti-AβScFv fused to the CH3 region, binds to the Aβ amyloid peptide of AD to cause disaggregation of amyloid in brain.

Figure 3. Immunocytochemistry of frozen sections of AD autopsy brain

The brain sections were immune stained with medium conditioned by COS cells that were transfected with pCD- ScFv (a, c), with medium conditioned by COS cells which were exposed only to Lipofectamine 2000 (b), or with the mouse IgG1 isotype control antibody (d). Magnification in panels a and b is the same, and the magnification bar in panel b is 88 μ m; magnification in panels c and d is the same and the magnification bar in panel d is 35 μ m.

Figure 4. SDS-PAGE and Western blotting of fusion antibody

(a) SDS-PAGE and Coomassie blue staining shows correct processing of intact fusion antibody by transfected COS cells. (b) Western blotting with an anti-human IgG primary antibody. The HIRMAb and the fusion antibody are comprised of the same light chain, which is 28 kDa. The size of the heavy chain of the HIRMAb is 55 kDa, whereas the size of the heavy chain of fusion antibody is 82 kDa. The heavy chain of the fusion antibody includes the 55 kDa heavy chain of the HIRMAb fused to the 27 kDa anti-A β ScFv.

Figure 5. Bi-functional binding of fusion antibody both to A β and to the human insulin receptor (a) The binding of [125 I]-murine anti-A β MAb to A β^{1-40} is competitively inhibited by either unlabeled murine anti-A β MAb or by the fusion antibody. The binding affinity of the fusion antibody is not significantly different from the affinity of the murine anti-A β MAb. (b) Binding of the genetically engineered HIRMAb or the fusion antibody to the HIR extracellular domain is measured with an ELISA. There is no binding of human IgG1 to the HIR ECD. The affinity of the fusion antibody for the HIR ECD is >50% of the affinity of the HIRMAb.

Figure 6. Blood to brain transport of fusion antibody in the primate

(a) The [¹²⁵I]-fusion antibody, and the [³H]-mouse anti-A β antibody, were injected intravenously into the adult Rhesus monkey and serum concentrations [% of injected dose (I.D.)/mL] were determined over a 3 hour period. There is no measurable clearance from blood of the [³H]-mouse anti-A β antibody during this time period, whereas the fusion antibody is rapidly cleared from blood, owing to uptake via the insulin receptor. (b) Brain volume of distribution (VD) of the [³H]-mouse anti-A β antibody and the [¹²⁵I]-fusion antibody in Rhesus monkey brain at 3 hours after a single intravenous injection. The VD for the homogenate and the post-vascular supernatant are shown. The VD for the [³H]-mouse anti-A β antibody, 10 uL/g, is equal to the brain arterial blood volume, which indicates this antibody is not transported across the primate BBB in vivo in the blood to brain direction. The VD for the [¹²⁵I]-fusion antibody is >100 uL/g in both the brain homogenate and the post-vascular supernatant, which indicates the [¹²⁵I]-fusion antibody is transported across the BBB.

Figure 7. Global distribution of fusion antibody to primate brain

Brain scans of adult Rhesus monkey at 3 hours after the intravenous administration of the [^{125}I]-fusion antibody demonstrates widespread distribution of the fusion antibody into the primate brain in vivo from blood. The top scan is the most frontal part of brain, and the bottom scan is the most caudal part of brain, and includes the cerebellum.

Figure 8. Brain to blood transport of fusion antibody in the rat

The [^{125}I]-fusion antibody was injected into the cortex under stereotaxic guidance, and the efflux of the fusion antibody from brain across the BBB was measured at 90 minutes after the injection. At this time nearly 60% of the injected fusion antibody has effluxed from brain. This efflux was >90% blocked ($p < 0.01$) by the co-injection of human Fc fragments, which indicates the efflux is mediated by an Fc receptor at the BBB. Data are mean $\pm$ S.E. ($n=4$ rats per point).

Figure 9. Fusion antibody disaggregates Abeta amyloid fibrils in vitro and in vivo

(a) Outline of Aβ plaque disaggregation assay. The Abeta fibrils are sandwiched by an anti-Abeta antibody that binds the carboxyl terminus (CT) of Abeta, and by the fusion antibody, or the murine anti-Abeta MAb, which binds the amino terminus (NT) of Abeta. (b) Disaggregation of Aβ fibrils in vitro by the fusion antibody. Aβ¹⁻⁴⁰ aggregates were formed over 6 days, followed by incubation with the fusion antibody, with human IgG1 (hIgG1), or with phosphate buffered saline (PBS) for either 1 or 4 hours at 37°C. The antibody that binds to the carboxyl terminal region of the Aβ¹⁻⁴⁰ peptide was plated in 96 well plates. The anti-Aβ ScFv portion of the fusion antibody binds to the amino terminal part of Aβ¹⁻⁴⁰. A positive ELISA signal is generated only if Abeta fibrils are present. The data show that the fusion antibody binds to Aβ¹⁻⁴⁰ plaque, and that this binding causes fibril disaggregation over a 4-hour period. (c) Amyloid plaque in brain of double transgenic APPswe/PS1dE9 mice stained with thioflavine-S. (d) The percent of brain occupied by amyloid plaque in either frontal cortex or hippocampus in the transgenic mice was quantitated with confocal microscopy. Amyloid was measured at 48 hours after the intra-cerebral injection of 20 pmol of fusion antibody into the frontal cortex and the hippocampus on one side of the brain. The amyloid burden in brain is expressed as a ratio of the % brain amyloid in the side ipsilateral to the injection relative to the % amyloid content in the contralateral frontal cortex or hippocampus, in the animals injected with either the fusion body, or with saline. Data are mean ± SE (n=3 mice for each treatment group). The decrease in amyloid content in either frontal cortex or hippocampus on the side of the fusion antibody injection, relative to the saline control, is statistically significant (*p<0.01).