Published in final edited form as:

Biotechnol Bioeng. 2010 February 15; 105(3): 627–635. doi:10.1002/bit.22576.

IgG-Single Chain Fv Fusion Protein Therapeutic for Alzheimer's Disease: Expression in CHO Cells and Pharmacokinetics and Brain Delivery in the Rhesus Monkey

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Abstract

Monoclonal antibodies (MAb) directed against the Abeta amyloid peptide of Alzheimer's disease (AD) are potential new therapies for AD, since these antibodies disaggregate brain amyloid plaque. However, the MAb is not transported across the blood-brain barrier (BBB). To enable BBB transport, a single chain Fv (ScFv) antibody against the Abeta peptide of AD was re-engineered as a fusion protein with the MAb against the human insulin receptor (HIR). The HIRMAb acts as a molecular Trojan horse to ferry the ScFv therapeutic antibody across the BBB. Chinese hamster ovary (CHO) cells were stably transfected with a tandem vector encoding the heavy and light chains of the HIRMAb-ScFv fusion protein. A high secreting line was isolated following methotrexate amplification and dilutional cloning. The HIRMAb-ScFv fusion protein in conditioned serum free medium was purified by protein A affinity chromatography. The fusion protein was stable as a liquid formulation, and retained high affinity binding of both the HIR and the Abeta amyloid peptide. The HIRMAb-ScFv fusion protein was radiolabeled with the ¹²⁵I-Bolton-Hunter reagent, followed by measurement of the pharmacokinetics of plasma clearance and brain uptake in the adult Rhesus monkey. The HIRMAb-ScFv fusion protein was rapidly cleared from plasma and was transported across the primate BBB in vivo. In conclusion, the HIRMAb-ScFv fusion protein is a new class of antibody-based therapeutic for AD that has been specifically engineered to cross the human BBB.

Keywords

blood-brain barrier; delivery systems; monoclonal antibody; Alzheimer's disease

Introduction

The dementia of Alzheimer's disease (AD) correlates with the accumulation in brain of amyloid plaque (Cummings and Cotman, 1995; Naslund et al, 2000). The brain amyloid of AD is comprised of the 40-43 amino acid Abeta amyloid peptide, and the intra-cerebral injection of a monoclonal antibody (MAb) against the Abeta peptide causes disaggregation of amyloid plaque, which is followed by the repair of dystrophic neurites (Lombardo et al, 2003). The drug development of anti-amyloid antibodies (AAA) for AD is limited by the lack of transport of the antibody therapeutic across the blood-brain barrier (BBB). One approach to the BBB drug delivery of recombinant proteins is the molecular Trojan horse technology, in which a non-transportable protein is fused to a BBB molecular Trojan horse (Pardridge, 2008). The

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latter is an endogenous peptide or peptidomimetic MAb that is transported on an endogenous BBB peptide receptor/transport system. The most potent BBB molecular Trojan horse is a MAb against the human insulin receptor (HIR), and HIRMAb fusion proteins have been engineered for neurotrophins (Boado et al, 2007a), enzymes (Boado et al, 2008), and decoy receptor biopharmaceuticals (Hui et al, 2009). However, when the non-transportable therapeutic is also a MAb, the problem becomes how to genetically engineer a fusion protein of the antibody therapeutic and the antibody delivery system. One approach is to produce a single chain Fv (ScFv) analogue of the therapeutic antibody, and fuse this ScFv to the carboxyl terminus of the delivery antibody. Previous studies have described the genetic engineering and transient expression in COS cells of a fusion protein of the chimeric HIRMAb and a ScFv antibody derived from a MAb directed against the Abeta amyloid peptide of AD (Boado et al, 2007b). This fusion protein is designated the HIRMAb-ScFv fusion protein, and the structure of the HIRMAb-ScFv fusion protein is shown in Figure 1. This fusion antibody is comprised of variable regions of the heavy chain (VH) and variable regions of the light chain (VL) from both the AAA and the HIRMAb, in addition to the constant (C)-region of human IgG1 heavy chain and human kappa light chain.

The purpose of the present study was to stably transfect Chinese hamster ovary (CHO) cells with a tandem vector encoding the heavy chain and light chain genes of the HIRMAb-ScFv fusion protein, as well as the gene encoding dihydrofolate reductase (DHFR), and to isolate a high producing CHO line secreting the HIRMAb-ScFv fusion protein in serum free medium. The in vitro stability of the fusion protein, and the binding of the fusion protein to both the HIR and to the Abeta peptide was examined. The HIRMAb-ScFv fusion protein fusion protein was injected intravenously into the adult Rhesus monkey, and the plasma pharmacokinetics and brain uptake was examined with both measurements of radioactivity and a specific sandwich ELISA that measures the immunoreactive HIRMAb-ScFv fusion protein in primate plasma.

Materials and Methods

Production of CHO line

The cDNA encoding the anti-Abeta ScFv was engineered following cloning of genes encoding the VH and VL of a murine antibody raised against an amino terminal portion of the Abeta peptide (Boado et al, 2007b). This ScFv was fused to the carboxyl terminus of the CH3 region of the heavy chain (HC) of the chimeric HIRMAb (Boado et al, 2007b). A tandem vector (TV) was engineered in which the expression cassettes encoding this fusion HC, as well as the HIRMAb light chain (LC), and the murine DHFR, are all contained on a single strand of DNA (Boado et al, 2007a). The TV was linearized and DG44 CHO cells were electroporated, followed by selection in hypoxanthine-thymine deficient medium and amplification with graded increases in methotrexate (MTX) up to 80 nM in serum free medium (SFM). The CHO line underwent 2 successive rounds of 1 cell/well dilutional cloning, and positive clones were selected by measurement of medium human IgG concentrations by enzyme-linked immunosorbent assay (ELISA). The CHO line was stable through multiple generations, and produced medium IgG levels of 10-20 mg/L in shake flasks at a cell density of 1-2 million cells/mL.

Protein A chromatography

The CHO cells were propagated in 1 L bottles, until 2.4L of conditioned serum free medium was collected. The medium was supplemented with 0.1% Tween-80, ultrafiltered with a 0.2 um Sartopore-2 sterile-filter unit (Sartorius Stedim Biotech, Goettingen, Germany), and applied to a 25 mL protein A Sepharose 4 Fast Flow (GE Life Sciences, Chicago, IL) column equilibrated in 25 mM Tris/25 mM NaCl/5 mM EDTA/pH=7.1/0.1% Tween-80. Following

application of the sample, the column was washed with 25 mM Tris/1 M NaCl/5 mM EDTA/pH=7.1/0.1% Tween-80, and the fusion protein was eluted with 0.1 M sodium acetate/pH=3.7/0.1% Tween-80. The acid eluate was pooled and neutralized to pH=5.6 with NaOH, and concentrated with an Ultra-15 microconcentrator (Millipore, Bedford, MA) and stored sterile-filtered at 4°C.

Analytical assays

The homogeneity of the HIRMAb-ScFv fusion protein was evaluated with sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) under both reducing and non-reducing conditions using a 4-12% Bis-Tris gel and the Nu-Page system (Invitrogen, Carlsbad, CA). Western blot analysis was performed with a goat anti-human IgG (H+L) antibody (Vector Labs, Burlingame, CA). The molecular size of the HIRMAb-ScFv fusion protein was determined with dynamic light scattering (DLS) using a Nicomp Model 380 Particle Analyzer (Agilent Technologies, Santa Barbara, CA).

ELISAs

HIR binding ELISA—The binding of the HIRMAb-ScFv fusion protein to the extracellular domain (ECD) of the HIR was determined by ELISA using CHO-derived HIR ECD affinity purified by lectin chromatography, as described previously (Coloma et al, 2000). The absorbance (A) values were fit to $A = A_{max}(S)/(ED50 + (S))$, by non-linear regression analysis, where A_{max} is the maximal absorbance, (S) is the concentration of the fusion protein, and ED50 is the concentration of fusion protein that yields a 50% increase in absorbance relative to A_{max} . The ED50 approximates the dissociation constant of the binding reaction between the fusion protein and the receptor.

A β ¹⁻⁴⁰ binding ELISA—The binding of the HIRMAb-ScFv fusion protein to the A β peptide was determined by ELISA. Recombinant streptavidin (SA) was obtained from Sigma Chemical Co. (St. Louis, MO), and plated in 96 wells overnight at 4°C in 100 μ L/well of 0.1 M NaHCO₃/pH=8.3 (400 ng/well). The SA was removed by aspiration, and the wells were blocked with 0.25% casein (Sigma) in 0.01 M Tris/0.15 M NaCl/7.4 (TBS) for 30 min at room temperature (RT). The wells were washed with TBS/0.05% Tween-20 (TBST) and 100 μ L/well of N-biotinyl A β ¹⁻⁴⁰ peptide (Invitrogen) was applied (1000 ng/well) for 60 min at RT. Following washing in TBST, 100 μ L/well of 30-3000 ng/mL of either HIRMAb-ScFv fusion protein or human IgG1 κ (Sigma) was incubated for 90 min at RT. A conjugate of alkaline phosphatase (AP) and a goat anti-human kappa light chain antibody (Sigma Chemical Co., St. Louis, MO) was applied in a volume of 100 μ L (170 ng)/well, followed by a 45 min incubation at RT. The wells were washed with TBST and the binding was measured colorimetrically with p-nitrophenylphosphate (Sigma).

HIR-IgG ELISA—The concentration of the HIRMAb-ScFv fusion protein in plasma was measured with a 2-site sandwich ELISA, which is similar in design to the HIR binding ELISA, except a murine MAb against the HIR is used to affinity purify the HIR ECD from the partially purified preparation produced following lectin affinity chromatography. The affinity purification step was introduced to increase the specificity of the assay to allow for measurement of the immunoreactive HIRMAb-ScFv fusion protein in plasma. A murine MAb against the HIR (Abcam, Cambridge, MA) was plated in 96-well plates overnight at 4°C in 0.1 M NaHCO₃/8.3 (100 ng/well); this antibody binds an epitope on the HIR that is spatially removed from the HIRMAb binding site. The solution was removed by aspiration, the wells were washed with 0.01 M Tris/0.15 M NaCl/7.4 (TBS) and 100 μ L (200 ng)/well of lectin affinity purified HIR ECD was added followed by a 90 min incubation at room temperature (RT). The wells were washed with TBS/0.05% Tween-20 (TBST), and either the plasma sample or the HIRMAb-ScFv reference standard was added in 100 μ L/well followed by a 60

min incubation at RT. A conjugate of AP and a goat anti-human kappa light chain antibody (Sigma) was applied in a volume of 100 μ L (170 ng)/well, followed by a 45 min incubation at RT. Following washing with TBST, 100 μ L/well of p-nitrophenylphosphate (Sigma) was incubated in the dark at RT for 15-30 min. The color development was terminated by the addition of 100 μ L/well of 1.2M NaOH, and color development was measured with an ELISA plate reader at 405 nm. The limit of detection was 1 ng/well of HIRMAb-ScFv fusion protein. The standard curve was determined with 0-1000 ng/mL solutions of HIRMAb-ScFv fusion protein and was curvilinear, which was fit with a non-linear regression analysis.

Radio-labeling of protein and primate administration

[125 I]-Bolton-Hunter reagent was purchased from American Radiolabeled Chemicals (St. Louis, MO). The HIRMAb-ScFv fusion protein was labeled to a specific activity of 7.8 μ Ci/ μ g and a trichloroacetic acid (TCA) precipitability of >96%. The TCA precipitation of the labeled HIRMAb-ScFv fusion protein remained >96% at 24 hours after iodination, and the HIRMAb-ScFv fusion protein was administered to the primate within 24 hrs of radio-labeling. Two adult female Rhesus monkeys, 6.4-6.9 kg, were obtained from Covance (Alice, TX). One animal was injected intravenously (IV) with 800 μ Ci of [125 I]-HIRMAb-ScFv fusion protein, which was co-injected with unlabeled HIRMAb-ScFv fusion protein, at a dose of 0.2 mg/kg. The second primate was injected with unlabeled HIRMAb-ScFv fusion protein at a dose of 1.0 mg/kg. Animals were initially anesthetized with intramuscular ketamine, and anesthesia was maintained by 1% isoflurane by inhalation. All procedures were carried out in accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the U.S. National Institutes of Health. Following intravenous drug administration, venous plasma was obtained at 1, 2.5, 5, 15, 30, 60, and 120 min for determination of radioactivity or immunoreactive HIRMAb-ScFv plasma concentration. Animals were euthanized, and samples of major organs (heart, liver, spleen, lung, kidney, skeletal muscle, and omental fat) were removed, weighed, and processed for determination of radioactivity. The cranium was opened and the brain was removed. Samples of frontal cortical gray matter, frontal cortical white matter, cerebellar gray matter, and cerebellar white matter were removed for radioactivity determination. Samples (~1 gram) of frontal cortex were removed for capillary depletion analysis, as described previously (Pardridge et al, 1995). For the capillary depletion analysis, the brain sample was divided into 3 fractions: total brain homogenate, post-vascular supernatant, and capillary pellet; the volume of distribution (VD) was determined for each of the 3 fractions from the ratio of total 125 I radioactivity in the fraction divided by the total 125 I radioactivity in the 120 min terminal plasma. A high VD in the post-vascular supernatant, as compared to the VD in the capillary pellet, is evidence for transport of the molecule through the BBB into brain parenchyma. Plasma and tissue samples were analyzed for 125 I radioactivity with a gamma counter (Wizard 1470, Perkin Elmer). The brain was cut into coronal slabs, and blocks were frozen in OCT embedding medium. Frozen sections (20 μ m) were cut with a cryostat at -15C; the sections were air dried and exposed to Kodak Biomax film for 2 weeks followed by xray film development. The films were scanned and the image was saved in Photoshop, and colorized with NIH Image software.

Pharmacokinetics

The trichloroacetic acid (TCA)-precipitable radioactivity in plasma, DPM/mL, was converted to % injected dose (ID)/mL, and the %ID/mL was fit to a bi-exponential equation,

$$\%ID/mL = A_1e^{-k_1t} + A_2e^{-k_2t}$$

The intercepts (A1, A2) and the slopes (k1, k2) were used to compute the median residence time (MRT), the central volume of distribution (Vc), the steady state volume of distribution (Vss), the area under the plasma concentration curve (AUC), and the systemic clearance (CL), as described previously (Pardridge et al, 1995). Non-linear regression analysis used the AR subroutine of the BMDP Statistical Software (Statistical Solutions Ltd, Cork, Ireland). Data were weighted by $1/(\%ID/mL)^2$. The organ clearance ($\mu L/min/g$), also called the permeability-surface area product, is computed from the terminal organ uptake ($\%ID/g$) and the 120 min plasma AUC ($\%IDmin/mL$) as follows:

$$\text{organ clearance} = [(\%ID/g)/AUC] * 1000$$

The brain clearance of the HIRMAb-ScFv fusion protein was compared to the brain clearance of human IgG1 reported previously (Boado and Pardridge, 2009). Since the IgG1 isotype control antibody has no receptor specificity and is not transported across the BBB, the brain clearance of the IgG1 isotype control antibody represents distribution in brain caused by sequestration within the cerebral plasma volume.

Results

A tandem vector was engineered, which contained the expression cassettes for the heavy chain fusion gene, the light gene, and the DHFR gene on a single strand of DNA. The 3 expression cassettes spanned 6,671 nucleotides (nt). The light chain was comprised of 234 amino acids (AA), which included a 20 AA signal peptide. The predicted molecular weight of the light chain is 23,398 Da with a predicted isoelectric point (pI) of 5.45. The fusion protein of the HIRMAb heavy chain and the anti-Abeta ScFv was comprised of 708 AA, which included a 19 AA signal peptide. The predicted molecular weight of the heavy chain, without glycosylation, is 75,580 Da with a predicted pI of 8.81. The domains of the fusion heavy chain include a 113 AA variable region of the heavy chain (VH) of the HIRMAb, a 330 AA human IgG1 constant (C)-region, a 2 AA linker (Ser-Ser), a 114 AA VH of the anti-Abeta ScFv, a 17 AA linker, and a 113 AA variable region of the light chain (VL) of the anti-Abeta ScFv. Single predicted N-linked glycosylation sequences were present in the second complementarity determining region (CDR) of the anti-Abeta ScFv VH, and in the human IgG1 C-region. The amino acid sequence of the fusion antibody expressed in CHO cells is identical to the sequence of the fusion antibody transiently expressed in COS cells (Boado et al, 2007b).

The CHO-derived HIRMAb-ScFv fusion protein was homogeneous on both non-reducing (Figure 2A) and reducing (Figure 2B) SDS-PAGE. The HIRMAb-ScFv fusion protein and the HIRMAb have identical light chains, and the difference in size of the heavy chain of the HIRMAb-ScFv fusion protein and the HIRMAb, ~30 kDa, is due to fusion of the ScFv to the heavy chain (Figure 1). The HIRMAb-ScFv fusion protein shows no aggregation on SDS-PAGE even after storage at 4C for 4 months; longer periods of storage were not tested. Western blot analysis showed the expected reactivity with both the light chain and the heavy chain of the HIRMAb-ScFv fusion protein (data not shown). No aggregation of the HIRMAb-ScFv fusion protein was detected with dynamic light scattering. The diameter of the HIRMAb-ScFv fusion protein was 28 ± 16 nm, which was proportional to the diameter of a fusion protein of the HIRMAb and glial derived neurotrophic factor (GDNF), 23 ± 6 nm, and the diameter of the HIRMAb, 17 ± 9 nm. A plot of the molecular weight of these 3 proteins vs the molecular diameter was linear (data not shown).

The design of the HIR binding ELISA is shown in Figure 3A; there is no binding to the HIR by human IgG1, while the HIRMAb-ScFv fusion protein shows saturable binding with and

ED50 of 1.9 ± 0.1 nM (Figure 3B). The design of the Abeta binding ELISA is shown in Figure 4A. There was no binding to Abeta by human IgG1, while the HIRMAb-ScFv fusion protein shows saturable binding with an ED50 of 2.0 ± 0.8 nM (Figure 4B).

The HIRMAb-ScFv fusion protein was radiolabeled with the ^{125}I -Bolton-Hunter reagent, and administered by intravenous (IV) injection into an adult Rhesus monkey with a dose of 0.2 mg/kg unlabeled HIRMAb-ScFv fusion protein (Methods). The clearance of the plasma radioactivity is shown in Figure 5. The concentration of the immunoreactive HIRMAb-ScFv fusion protein was measured with the sandwich ELISA (Methods), which produced a curvilinear standard curve with the HIRMAb-ScFv fusion protein reference standard, as shown in Figure 6. This assay was used to determine the concentration of immunoreactive HIRMAb-ScFv fusion protein in Rhesus monkey plasma, and the concentrations are shown in Table I. The plasma concentrations were divided by the injected dose (ID) to produce the % ID/mL for the immunoreactive HIRMAb-ScFv fusion protein, and these data are plotted in Figure 5. The plasma decay profiles for the radioactive HIRMAb-ScFv fusion protein and the immunoreactive HIRMAb-ScFv fusion protein are comparable. The plasma radioactivity decay curve was fit to a bi-exponential equation to yield the pharmacokinetic (PK) parameters shown in Table II. A PK analysis based on the immunoreactive HIRMAb-ScFv fusion protein concentrations yielded PK parameters comparable to those shown in Table II.

The uptake of the ^{125}I -HIRMAb-ScFv fusion protein by brain and peripheral organs was expressed as %ID/100 gram tissue at 2 hours after IV injection (Table III). The brain uptake of the ^{125}I -HIRMAb-ScFv fusion protein was also measured with film autoradiography of frozen coronal sections of brain removed 2 hours after IV injection of the fusion protein. There is global uptake of the fusion protein by brain with higher uptake in gray matter as compared to white matter (Figure 7). The brain clearance of the ^{125}I -HIRMAb-ScFv fusion protein at 2 hours after IV injection, 0.50 ± 0.02 uL/min/gram, is increased in comparison with the brain clearance of a human IgG1 vascular space marker, 0.11 ± 0.01 uL/min/gram (Boado and Pardridge, 2009). Transport of the ^{125}I -HIRMAb-ScFv fusion protein through the BBB in vivo was confirmed with the capillary depletion method. The 2 hour brain volume of distribution (VD) in the brain homogenate, the pos-vascular supernatant, and the micro-vascular pellet was 50 ± 3 , 42 ± 2 , and 2.9 ± 0.7 uL/gram, respectively.

The linearity of the plasma PK parameters of the HIRMAb-ScFv fusion protein was examined by injection of a 5-fold larger dose, 1.0 mg/kg, of the fusion protein in an adult Rhesus monkey, and the plasma concentrations of the immunoreactive HIRMAb-ScFv fusion protein are shown in Table I. These data were fit to the bi-exponential model (Methods). The systemic clearance of the immunoreactive HIRMAb-ScFv fusion protein was 1.69 ± 0.07 mL/min/kg and 1.32 ± 0.03 mL/min/kg, respectively, after injection of 0.2 and 1.0 mg/kg fusion protein. There was a linear relationship between the plasma AUCss and dose, as the AUCss was 118 ± 5 ug•min/mL and 759 ± 18 ug•min/mL, respectively, after the IV injection of 0.2 and 1.0 mg/kg fusion protein.

Discussion

The results of this study are consistent with the following conclusions. First, an IgG-ScFv fusion protein has been engineered and a high expressing CHO cell line cultured in serum free medium has been cloned (Methods). Second the HIRMAb-ScFv fusion protein is stable without aggregation based on SDS-PAGE (Figure 2), Western blotting, and dynamic light scattering (Results). Third, the HIRMAb-ScFv fusion protein is bi-functional and binds both the HIR and the Abeta peptide of AD with high affinity (Figures 3 and 4). Fourth, the HIRMAb-ScFv fusion protein is rapidly cleared from plasma in the adult Rhesus monkey (Figure 5, Table I), and the PK profile shows a linear relationship between dose and plasma AUC (Results). Fifth, the

HIRMAb-ScFv fusion protein penetrates the BBB in the Rhesus monkey based on film autoradiography (Figure 7), organ uptake (Table III), and quantitative measurements of BBB clearance (Results).

The background of the present work is derived from two unrelated fields: the development of MAb-based therapies for AD that disaggregate brain amyloid, and the engineering of fusion proteins comprised of 2 unrelated MAb molecules. With regard to the MAb drug development for AD, the present work is based on the hypothesis that an anti-Abeta antibody (AAA) therapeutic for AD must be re-engineered to create a trifunctional molecule with the following properties: (i) receptor-mediated influx from blood to brain across the BBB, (ii) MAb-mediated disaggregation of the Abeta amyloid plaque, which resides in brain behind the BBB, and (iii) receptor-mediated efflux of the AAA from brain back to blood, so as to result in a net decrease in the Abeta amyloid burden in the brain in AD (Boado et al, 2007b). Prior AAAs developed as therapeutics for AD only disaggregate amyloid plaque and have not been engineered with respect to either influx or efflux across the BBB (Jicha, 2009). In the case of the HIRMAb-ScFv fusion protein, the HIRMAb part of the fusion protein binds the HIR on the BBB to trigger receptor-mediated influx across the BBB (Boado et al, 2007b). The C-region of the HIRMAb contains the CH2-CH3 interface, which is the binding site for the neonatal Fc receptor (FcRn), and prior work has demonstrated receptor-mediated efflux of the HIRMAb-ScFv fusion protein via the BBB FcR in the rat (Boado et al, 2007b). The ScFv part of the HIRMAb-ScFv fusion protein binds and disaggregates brain amyloid, as demonstrated previously in double transgenic AD mice (Boado et al, 2007b).

There are multiple approaches for the engineering of a fusion protein comprised of 2 unrelated antibodies (Marvin and Zhu, 2005), with the goal being secretion by the transfected host cell of a single molecular species. In the present work, the VH and VL of the anti-Abeta antibody were separately cloned to form a ScFv, which was then fused to the carboxyl terminus of the chimeric HIRMAb (Figure 1). This approach places the ScFv in a dimeric configuration. The fusion of the ScFv at the carboxyl terminus of the heavy chain eliminates any steric hindrance at the amino terminal part of the HIRMAb, which binds to the HIR. The result is that the HIRMAb-ScFv fusion protein is secreted in high amounts by transfected CHO cells, and the purified fusion protein is a stable, bi-functional molecule. The HIRMAb-ScFv fusion protein binds both the HIR and the Abeta peptide with low nM binding constants (Figures 3 and 4). The ED50 of the HIRMAb-ScFv fusion protein binding to the HIR, 1.9 ± 0.1 nM (Figure 3), is higher than the ED50 of the HIRMAb, which is 0.53 ± 0.02 nM (Boado et al, 2007b), and this lower affinity is attributed to the effects of fusion of the ScFv to the HIRMAb. Nevertheless, the affinity of the fusion protein for the HIR is still in the low nM range. The ED50 of HIRMAb-ScFv fusion protein binding to the Abeta peptide, 2.0 ± 0.8 nM (Figure 4), is in the low nM range, which is indicative of relatively high affinity. In contrast, the affinity of many single chain antibodies is low, owing to the monovalency of the single chain domain (Holliger and Hudson, 2005). The high avidity of the ScFv moiety for the Abeta peptide is attributed to the placement of the ScFv in a bivalent conformation following fusion to the HIRMAb (Figure 1).

Single chain antibodies, owing to the reduced size, have increased uptake by peripheral tumors (Holliger and Hudson, 2005). However, ScFv antibodies are still too large to cross the BBB, where transport is restricted when the size of the molecule exceeds 400 Da (Pardridge, 2008). The HIRMAb-ScFv fusion protein is rapidly removed from plasma following IV injection in adult Rhesus monkeys (Figure 5). This rapid clearance is to be compared and contrasted to the rate of clearance of the separate antibody entities: the HIRMAb and the original 150 kDa anti-Abeta MAb. The HIRMAb is rapidly removed from plasma in the primate owing to receptor-mediated uptake by tissues, including brain, with a systemic clearance ranging from 0.22 to 1.0 mL/min/kg (Coloma et al, 2000), which approximates the clearance of the HIRMAb-ScFv fusion protein, 1.72 ± 0.08 mL/min/kg (Table II). In contrast, the anti-Abeta MAb is removed

so slowly from plasma in the Rhesus monkey that clearance estimates cannot be made in a 2-hour experimental time period (Boado et al, 2007b). During the first 2 hours after injection, there is no measureable removal of the anti-Abeta MAb from plasma, because the MAb has no receptor-specificity that would enhance the uptake by organs, including brain. The slow removal of the anti-Abeta MAb from blood results in sustained, high plasma concentrations of the antibody, but this may have a deleterious effect on the antibody safety profile. The administration of anti-Abeta MAb's results in a redistribution of Abeta stores, most likely from platelets, such that the plasma concentration of Abeta increases to nearly 1 uM (Wilcock et al, 2006). Plasma concentrations of Abeta of this magnitude are associated with BBB disruption (Jancso et al, 1998; Su et al, 1999), and cerebral micro-hemorrhage is a consistent side effect of Abeta MAb therapy in AD (Pfeiffer et al, 2002; Wilcock et al, 2004). The rapid removal of the HIRMAb-ScFv fusion protein from plasma is observed at both 0.2 and 1.0 mg/kg doses of the fusion protein (Table I). The rapid removal of the HIRMAb-ScFv fusion protein is due to uptake by peripheral organs, primarily liver and spleen (Table III).

The HIRMAb-ScFv fusion protein penetrates the BBB in the adult Rhesus monkey as demonstrated by the film autoradiography of brain (Figure 7), the capillary depletion methods (Results), and the estimate of the BBB permeability coefficient (Results). The brain uptake of the HIRMAb-ScFv fusion protein is 0.30 ± 0.01 %ID/100 g brain. The Rhesus monkey brain weighs approximately 100 grams (Davies and Morris, 1993). Therefore, the concentration of the HIRMAb-ScFv fusion protein in brain following the IV injection of 1 mg/kg is projected to be 210 ng/gram brain, which is equivalent to 1 pmol/gram brain, or 1.4 nM. This fusion antibody concentration in brain approximates the ED50 of fusion antibody binding to either the HIR (Figure 3) or the Abeta peptide (Figure 4). Such concentrations cause a net clearance of brain amyloid plaque, as prior work has shown that the injection of 20 pmol of the HIRMAb-ScFv fusion protein into the brain of AD transgenic mice causes a rapid 40% removal of cortical or hippocampal amyloid plaque within 48 hrs of a single administration of the fusion protein (Boado et al, 2007b).

In conclusion, the present work demonstrates the feasibility of expression and purification of an IgG-ScFv fusion protein produced by CHO cells cultured in serum free medium. The HIRMAb-ScFv fusion protein binds the HIR and Abeta with high affinity, is rapidly removed from plasma following IV administration and penetrates the BBB in vivo. HIRMAb-based fusion proteins have recently been shown to have excellent safety profiles, and to cause no changes in glycemic control even after high 20 mg/kg doses (Pardridge and Boado, 2009; Boado et al, 2009). IgG-ScFv fusion proteins may also prove to have weak immunogenicity. Recent work shows that amino acid sequences within the C-region of human IgG1, called Tregitopes, induce immune tolerance. (DeGroot et al, 2008). Therefore, re-engineering ScFv-based antibody drugs as IgG fusion proteins may reduce the immunogenicity of the recombinant protein.

Acknowledgments

Winnie Tai and Phuong Tram provided technical assistance. This work was supported by NIH grant R43-MH083334.

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HIR binding

Abeta binding

Figure 1.

The HIRMAb-ScFv fusion antibody is formed following fusion of an anti-Abeta peptide ScFv to the carboxyl terminus of the CH3 region of the heavy chain of the chimeric HIRMAb. Fusion of the ScFv to the heavy chain carboxyl region restores the ScFv to a bivalent structure. The fusion antibody is a hetero-tetrameric molecule comprised of 2 heavy chains and 2 light chains. The light chains are derived from the HIRMAb.

Figure 2. (A) SDS-PAGE under non-reducing conditions for the chimeric HIRMAb (lane 1), the HIRMAb-ScFv fusion protein after 4 months storage at 4°C (lane 2), and the HIRMAb-ScFv fusion protein reference standard stored at -20°C. (B) SDS-PAGE under reducing conditions for the HIRMAb-ScFv fusion protein reference standard (lane 1), the HIRMAb-ScFv fusion protein after 4 months storage at 4°C (lane 2), and the chimeric HIRMAb (lane 3).

Figure 3.

(A) The ELISA used to measure HIRMAb-ScFv binding to the HIR employs the HIR ECD as the capture reagent and an anti-human light chain kappa (hLC^K) conjugate of alkaline phosphatase (AP) as the detector reagent. (B) Binding of the HIRMAb-ScFv fusion protein to the HIR is saturable, whereas there is no binding to the HIR of human (h) IgG1K. The concentration that gives 50% binding, ED50, was computed by non-linear regression analysis.

Figure 4.

(A) The ELISA used to measure HIRMAb-ScFv binding to the $A\beta^{1-40}$ employs a complex of streptavidin (SA) and N-biotinyl $A\beta^{1-40}$ as the capture reagent and an anti-human light chain kappa (hLC^K) conjugate of alkaline phosphatase (AP) as the detector reagent. (B) Binding of the HIRMAb-ScFv fusion protein to the $A\beta^{1-40}$ is saturable, whereas there is no binding to the $A\beta^{1-40}$ of human (h) IgG1 κ . The concentration that gives 50% binding, ED50, was computed by non-linear regression analysis.

Figure 5.

Plasma concentration of the HIRMAb-ScFv fusion protein after injection of an 0.2 mg/kg dose in the Rhesus monkey was determined by either radioactivity counting, or by immunoreactivity using the assay outlined in Figure 5A. Data are expressed as % of injected dose (I.D.)/mL.

Figure 6.

Binding of the HIRMAb-ScFv fusion protein to the HIR is saturable, whereas there is no binding to the HIR of human (h) IgG1K. This curve exemplifies the standard curve used in the assay to determine the concentration of immunoreactive HIRMAb-ScFv fusion protein in primate plasma (Table III). Curve fitting produced a half-maximal concentration (EC50) of 120 ± 36 ng/mL and a maximal binding (Bmax) of 0.93 ± 0.09 .

Figure 7. Film autoradiography of Rhesus monkey brain removed 2 hours after an intravenous administration of [125 I]-HIRMAb-ScFv fusion protein. Coronal sections through the cerebrum (top panel), and hindbrain/cerebellum (bottom panel) are shown.

Table I

Plasma concentration (ng/mL) of immunoreactive HIRMAb-ScFv fusion protein in the Rhesus monkey

Time (minutes)	Injection dose	
	0.2 mg/kg	1.0 mg/kg
1	1,877 ± 107	17,322 ± 990
2.5	1,549 ± 120	14,992 ± 632
5	1,457 ± 91	12,682 ± 235
15	1,138 ± 56	7,364 ± 43
30	900 ± 77	5,717 ± 221
60	663 ± 24	4,239 ± 230
120	387 ± 22	1,975 ± 166

Table IIPharmacokinetic parameters of [125 I]-HIRMAb-ScFv clearance from plasma

name	units	parameter
A1	%ID/mL	0.157 ± 0.005
A2	%ID/mL	0.051 ± 0.004
k1	min ⁻¹	0.090 ± 0.006
k2	min ⁻¹	0.0068 ± 0.0007
MRT	min	120 ± 11
Vc	mL/kg	75 ± 2
Vss	mL/kg	206 ± 12
AUC ₀₋₁₂₀	%ID•min/mL	5.9 ± 0.1
AUC _{ss}	%ID•min/mL	9.1 ± 0.4
AUC _{ss}	μg•min/mL	128 ± 6
CL	mL/min/kg	1.72 ± 0.08

Determined from the plasma radioactivity data in Figure 5 at an injection dose of fusion protein of 0.2 mg/kg. A1 and A2, and k1 and k2, are the intercepts and slopes, respectively, of the 2 exponential functions describing fusion antibody clearance from plasma. MRT=median residence time; Vc=central volume of distribution; Vss=steady state volume of distribution; AUC₀₋₁₂₀ is the plasma area under the concentration curve between 0 and 120 min after injection; AUC_{ss} is the steady state AUC; and CL is the systemic clearance rate of the fusion antibody.

Table IIIOrgan uptake of [125 I]-HIRMAb-ScFv in the Rhesus monkey

organ	organ uptake (%ID/100g)
Frontal gray	0.30 ± 0.01
Frontal white	0.18 ± 0.01
Cerebellar gray	0.32 ± 0.02
Cerebellar white	0.23 ± 0.03
heart	1.2 ± 0.1
liver	9.8 ± 0.2
spleen	10.6 ± 0.1
lung	2.9 ± 0.1
Skeletal muscle	0.31 ± 0.07
fat	1.1 ± 0.1

Data are mean $\pm$ SE, and organs were removed 2 hours after the IV injection of the labeled fusion protein co-injected with 0.2 mg/kg HIRMAb-ScFv fusion protein.