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Muscle-directed anti-A β single-chain antibody delivery via AAV1 reduces cerebral A β load in an Alzheimer's disease mouse model

Junling Yang¹, Abhinandan Pattanayak¹, Min Song^{1,2}, Jinghong Kou¹, Hiroaki Taguchi³, Sudhir Paul³, Selvarangan Ponnazhagan⁴, Robert Lalonde⁵, and Ken-ichiro Fukuchi^{1,*}

¹Department of Cancer Biology and Pharmacology, University of Illinois College of Medicine at Peoria, P.O. Box 1649, Peoria, IL 61656, USA

²Department of Neurology, College of Medicine, JiuJiang University, Jiangxi, PR China

³Chemical Immunology Research Center, Department of Pathology and Laboratory Medicine, University of Texas Houston Medical School, Houston, Texas 77030 USA

⁴Pathology, Schools of Medicine and Dentistry, University of Alabama at Birmingham, Birmingham, Alabama 35294-0024, USA

⁵Department Psychology, ICONES Laboratory, University of Rouen, EA 4699, 76821 Mont-Saint-Aignan, France

Abstract

We previously reported that anti-A β single-chain antibody (scFv59) brain delivery via recombinant adeno-associated virus (rAAV) was effective in reducing cerebral A β load in an AD mouse model without inducing inflammation. Here, we investigated the prophylactic effects and mechanism of a muscle-directed gene therapy modality in an AD mouse model. We injected rAAV serotype 1 encoding scFv59 into the right thigh muscles of 3-month-old mice. Nine months later, high levels of scFv59 expression were confirmed in the thigh muscles by both immunoblotting and immunohistochemistry. As controls, model mice were similarly injected with rAAV1 encoding anti-HIV Gag antibody (scFvGag). AAV1-mediated scFv59 gene delivery was effective in decreasing A β deposits in the brain. Compared with the scFvGag group, levels of A β in cerebrospinal fluid (CSF) decreased significantly while A β in serum tended to increase in the scFv59 group. AAV1-mediated scFv59 gene delivery may alter the equilibrium of A β between the blood and brain, resulting in an increased efflux of A β from the brain owing to antibody-mediated sequestration/clearance of peripheral A β . Our results suggest that muscle-directed scFv59 delivery via rAAV1 may be a prophylactic option for AD and that levels of CSF A β may be used to evaluate the efficacy of anti-A β immunotherapy.

Keywords

single-chain antibody; adeno-associated virus; amyloid; Alzheimer's disease; cerebrospinal fluid

*Corresponding author: K. Fukuchi; phone: 309-671-8545, Fax: 309-671-8403, kfukuchi@uic.edu.

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Introduction

Alzheimer's disease (AD) is the most common neurodegenerative disease affecting about 10% of the population over 65 and 40% of those over 85 years of age (Morgan 2011). Cardinal pathological changes of AD are extracellular amyloid-beta ($A\beta$) deposits and intracellular neurofibrillary tangles containing hyperphosphorylated tau in the brain. $A\beta$ is produced by the proteolytic processing of the amyloid precursor protein (APP) (Thinakaran and Koo 2008). Although the precise etiology of AD is unclear, the amyloid hypothesis has been very influential in pathogenesis and drug development (Karran et al. 2011). The main claim of the hypothesis is that deposition/accumulation of $A\beta$ in the brain is the primary event leading to dementia in AD. Therefore, many studies attempted to decrease $A\beta$ deposits in the brain by limiting production of $A\beta$, removing $A\beta$ from the brain through its proteolytic degradation or immunotherapeutic mobilization, or enhancing efflux transport across the blood-brain barrier. Because oligomeric aggregates of $A\beta$ are proposed to be neurotoxic, attempts have also been made to alleviate the toxicity of $A\beta$ aggregates (Grill and Cummings 2010; Hardy 2006; Kasturirangan and Sierks 2010).

In the last decade, vaccination to reduce $A\beta$ deposits has received more attention than any other therapeutic approaches in AD. Schenk et al. (1999) reported that immunization of APP transgenic mice with synthetic $A\beta$ by repeated needle injection prevented or reduced $A\beta$ deposits. The subsequent experiments demonstrated that their memory and learning deficits were improved by $A\beta$ vaccination (Janus et al. 2000; Morgan et al. 2000). However, human clinical trials of $A\beta$ immunization (AN1792) were halted due to brain inflammation presumably induced by T-cell-mediated and/or Fc-mediated immune responses (Check 2002; Orgogozo et al. 2003). Thus, it is crucial to find a safe and effective immune therapy. Peripheral administration of anti- $A\beta$ antibodies reduced $A\beta$ deposits in an AD mouse model despite modest serum levels of antibodies, indicating that an active T-cell-mediated immune response is unnecessary (Bard et al. 2000). Because anti- $A\beta$ antibodies that are capable of crossing the blood-brain barrier were bound to $A\beta$ deposits in the brain and increased $A\beta$ -uptake by microglia after $A\beta$ immunization (Nicoll et al. 2006; Schenk et al. 1999), Fc-mediated phagocytosis by microglia was originally postulated as an $A\beta$ clearance mechanism. However, clearance of $A\beta$ deposits by $A\beta$ immunization in a Fc receptor-knockout AD mouse model is equivalent to that in a Fc receptor-sufficient AD mouse model, indicating that clearance of $A\beta$ deposits by $A\beta$ immunization is not dependent on Fc-receptor-mediated phagocytosis (Das et al. 2003). DeMattos et al. (2001) showed that sequestration of plasma $A\beta$ by an anti- $A\beta$ antibody can reduce brain $A\beta$ load in an AD mouse model without binding of the antibody to $A\beta$ deposits, demonstrating that $A\beta$ immunotherapy can reduce cerebral $A\beta$ load by altering the peripheral $A\beta$ levels. Thus, anti- $A\beta$ single-chain variable fragment antibody (scFv) with the advantage of lacking Fc will reduce the chance of a cellular immune response mediated by Fc receptors without compromising its capacity of $A\beta$ clearance. Previous studies found intracranial injection with anti- $A\beta$ scFvs is effective in reducing cerebral $A\beta$ load in AD mouse models (Fukuchi et al. 2006a; Fukuchi et al. 2006b; Ryan et al. 2010). Wang et al. (2009; 2010) demonstrated that intramuscular delivery of an anti- $A\beta$ scFv gene in an AD mouse model reduced amyloid deposits and ameliorated its learning and memory deficits without inducing discernible inflammation, brain microhemorrhage, and neutralizing antibodies against the scFv. However, the mechanism by which $A\beta$ deposits were removed from the brain via muscle-directed anti- $A\beta$ scFv gene delivery is still unclear. In this study, we investigate the prophylactic effects and mechanism of a muscle-directed gene therapy modality by recombinant adeno-associated virus (rAAV) vector encoding scFv59 (a specific anti- $A\beta$ scFv that showed the highest immunoreactivity to $A\beta$ plaques among anti- $A\beta$ scFvs isolated in our lab) on prevention of $A\beta$ deposits.

Materials and Methods

Recombinant adeno-associated virus preparations

Expression of plasmid vectors, pAAV-CAscFv59 and pAAV-CAscFv-Gag, for rAAV production were described previously (Fukuchi et al. 2006b). Briefly, cDNAs encoding scFvs were placed under the control of cytomegalovirus enhancer/ β -actin promoter in a rAAV expression plasmid, pAAV-CAscFv. In the plasmid vectors, the Kozak sequence and woodchuck hepatitis virus post-transcriptional regulatory element were included to increase the translation efficiency. FLAG-His tag was placed at the C-terminal ends of scFvs as a marker. pAAV-CAscFv59 encodes scFv59 that reacts with oligomeric A β as well as A β deposits in the brain (Fukuchi et al. 2006a). pAAV-CAscFv-Gag encodes scFv-Gag that binds to the human immunodeficiency virus (HIV) Gag protein (Fukuchi et al. 2006b). Using the calcium phosphate transfection method as previously described (Grimm et al. 2003), HEK293 cells were transfected with pAAV-CAscFv59 and one of the following helper plasmids, pDP1, pDG, and pDP6 to produce pseudotyped rAAV1, rAAV2, and rAAV6 encoding scFv59, respectively. The helper plasmids carried both the AAV2 *rep2* gene and one of the serotype specific *cap* genes (Grimm et al. 2003). Produced viral particles were released from the cells by rapid freeze and thaw and purified by iodixanol gradient centrifugation. The iodixanol gradient fraction was further purified by HPLC using a 5-ml HiTrap Q column (GE Healthcare, Piscataway, NJ) as described before (Zolotukhin et al. 2002). Pseudotyped rAAV8 encoding scFv59 was similarly prepared by use of p5E18-VD2/8 bearing the AAV2 *rep2* and AAV8-Cap gene. A control rAAV1 encoding scFv-Gag was similarly prepared, also. The titers of rAAV virions that contained the vector genomes were determined by the quantitative dot-blot assay as described previously (Fukuchi et al. 2006b).

Experimental animals and muscular injection of rAAV-scFv59

C57BL/6 mice (6–8 weeks old) purchased from Jackson Laboratory (Bar Harbor, ME) were used to optimize intramuscular delivery of scFv59 by testing 4 differently pseudotyped rAAVs. Mice were randomly assigned to 5 treatment groups in such a manner as there was no significant intergroup difference in body weight: PBS, rAAV1-CAscFv59, rAAV2-CAscFv59, rAAV6-CAscFv59 and rAAV8-CAscFv59 ($n=10$ for each group). Mice were anesthetized by pentobarbital and injected with rAAV [2.5×10^{11} vector genomes (vg) in 100 μ l PBS/mouse] into the quadriceps muscle of each right leg using a 36 gauge needle. Control mice received the same amount of PBS. Three months after the rAAV injection, the experimental mice were terminated by a lethal injection of sodium pentobarbital to determine expression levels of scFv59 in the muscle. An AD mouse model, B6.Cg-Tg (APP^{swe}, PSEN1^{dE9}) 85Dbo/J mice (TgAPP^{swe}/PS1^{dE9} mice) purchased from Jackson Laboratory (Jankowsky et al. 2004), was used to study the prophylactic effects of muscle-directed rAAV-CAscFv59. Three-month old TgAPP^{swe}/PS1^{dE9} male mice were divided into 3 groups: 1) mice subjected to a single injection of rAAV-CAscFv59 (2.5×10^{11} vg in 100 μ l PBS/mouse) into the quadriceps muscle of their right legs (scFv59 group, $n = 10$), 2) mice subjected to a single injection of rAAV-CAscFv-Gag (2.5×10^{11} vg in 100 μ l PBS/mouse) into the quadriceps muscle of their right legs (Gag group, $n = 6$), and 3) mice subjected to a single injection of 100 μ l PBS into the quadriceps muscle of their right legs (PBS group, $n = 2$).

Immunohistochemical and histochemical analyses

Nine months after rAAV-CAscFv59 muscle injection, mice were deeply anesthetized with pentobarbital and cardinally perfused with cold PBS and the brains were quickly removed. The neocortices and hippocampi of the right hemispheres were separately dissected and stored in -80°C for further studies. The left hemispheres were fixed in 4%

paraformaldehyde for 16 h and then stored overnight in 30% sucrose in 0.1M PBS and frozen in Tissue-Tech optimal cutting temperature compound. Frozen sections (35 μ m thick) were prepared for immunohistochemical staining to determine the brain A β load (6E10 antibody) and the activation of microglia (anti-Iba1 and CD11b antibody) using the avidin-biotin-peroxidase method (Vectastatin ABC kit, Vector, Burlingame, CA). Endogenous peroxidase was eliminated by treatment with 3% H₂O₂/10% methanol Tris-buffered saline (TBS) for 1 h at room temperature. After washing with 0.1M TBS (PH 7.4), sections were blocked with blocking buffer for 1 h at room temperature to prevent non-specific protein binding. The sections were then incubated with primary antibodies, 6E10 (1:1000; Signet Laboratories, Dedham, MA), anti-CD11b (1:200, Serotec, MCA711, Raleigh, NC) or anti-Iba1 (1:1000, Wako, Richmond, VA), overnight at 4°C. The sections were rinsed in 0.1 TBST and incubated with appropriated biotinylated secondary antibodies for 1 h at room temperature. Finally, the avidin-biotin-peroxidase method using 3,3'-diaminobenzidine (DAB) as a substrate (Vector, Burlingame, CA) was performed according to manufacturer's protocol. Fibrillar A β plaques were stained with thioflavin S. In brief, sections were stained with 1% thioflavin S for 5 min followed by destaining in 70% ethanol. Histomorphometry for quantification of amyloid deposition, astrocyte and microglial activation was performed using an Olympus BX61 automated microscope, Olympus Fluoview system, and Image Pro Plus v4 image analysis software (Media Cybernetics, Silver Spring, MD) capable of color segmentation and automation via programmable macros. For each mouse, 4–5 coronal brain sections starting at 1.2 mm posterior to the bregma to caudal, each separated by approximately 300 μ m intervals, were analyzed. Areas stained with specific antibodies were expressed as percentages of total hippocampus or neocortex examined.

The quadriceps muscle injected with rAAV-CAscFv59 were fixed in 4% paraformaldehyde for 16 h and embedded in paraffin to detect scFv59 expression by immunohistochemistry. The sections were mounted on slides and dewaxed with xylene and gradually hydrated. Antigen retrieval was achieved by pressure cooking in Antigen Retrieval Citra Solution reagent (BioGenex, Fremont, CA) for 15 min. After allowing slides to cool for 1–2 h at room temperature, slides were incubated with anti-M2 flag (Sigma, St. Louis, MO) antibody (1:100 dilution) in blocking buffer overnight at 4°C. Then, the slides were incubated with biotinylated secondary antibody for 1 h and visualized by the avidin-biotin-peroxidase method and DAB. The slides were counterstained with hematoxylin.

Determination of anti-scFv59 antibodies in sera

Blood samples were collected at 2, 4, 6, and 8 months after rAAV-CAscFv59 muscle injection by cutting mice tail tips. Briefly, approximately 100 μ l of blood per mouse was drawn from the tail vein, incubated at room temperature for 1 h, and then transferred to 4°C. After overnight incubation, blood was centrifuged at 12,000 $\times$ g for 30 min and the serum was stored at –80°C and thawed at the time of assay. Enzyme-linked immunosorbent assay (ELISA) was carried out to determine titers of anti-scFv antibodies. Briefly, 96-well plates were coated with 500 ng purified scFv59 per well at 4°C overnight, followed by incubation with blocking buffer (1x PBS containing 0.5% BSA, 0.05% Tween-20 and 5% goat serum) at room temperature for 1 h. Then, diluted serum samples (1:50) were added to microtiter wells and incubated at 4°C overnight. The next day, the microplates were washed 5 times using washing buffer (1x PBS containing 0.05% Tween-20), and then incubated with horseradish peroxidase-conjugated secondary antibody at room temperature for 1 h. The microplates were then washed with washing buffer 5 times followed by incubation with 3,3',5,5'-tetramethylbenzidine (TMB) (Kirkegaard & Perry Laboratories Inc., Gaithersburg, MD) for 15 min to allow the development of color. The reaction was stopped by adding 100 μ l of 1 N H₂SO₄. The optic densities were determined at 450 nm using a Microplate Reader.

Serial dilutions of anti-FLAG M2 antibody were used as standard to determine titers of anti-scFv antibodies.

Murine CSF isolation

Cerebrospinal fluid was isolated from the cisterna magna compartment using the method described by DeMattos et al. (2002). Mice were anesthetized by pentobarbital and fixed face down on a narrow platform. An incision was made from the top of the skull to the dorsal thorax. The musculature from the base of the skull to the first vertebrae was carefully removed to expose the meninges overlying the cisterna magna. The surrounding area was gently cleaned with 1x PBS using cotton swabs to remove any residual blood or other interstitial fluid. The arachnoid membrane covering the cistern was punctured with a 29 gauge insulin syringe. A polypropylene narrow bore pipette was immediately placed in the hole to collect CSF. As the primary CSF exiting the compartment was collected, a second collection was performed after the cistern was refilled within 2 min. About 10 to 15 μ l CSF was collected from each mouse.

Quantification of brain A β , CSF, and serum by ELISA

The neocortex and hippocampus were removed from -80°C , lysed using the Bio-Plex cell lysis kit (Bio-Rad Laboratories, Hercules, CA) and homogenized according to the manufacturer's protocol, and centrifuged at $16,000 \times g$ for 30 min at 4°C . The supernatants containing buffer soluble A β were collected and the protein concentrations in the supernatants were determined by Bio-Rad Protein Assay (Bio-Rad). The pellets containing insoluble A β were further dounce homogenized in guanidine hydrochloride (final concentration, 5 M) and then rock-shaken for 3–4 h at room temperature. Levels of buffer-soluble and insoluble A β in the neocortex and hippocampus, and A β in the CSF and serum were quantified by the A β 42 and A β 40 ELISA kits (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol.

Immunoblotting for detection of scFv in muscle

The quadriceps muscles were homogenized in 2x Laemmli buffer (final concentration: 60mM Tris-HCl, 2% SDS, 10% glycerol, 5% 2-mercaptoethanol, 0.001% bromphenol blue, pH: 6.8) and protein concentration was determined by Bio-Rad protein assay. Fifty μ g of protein from each sample were subjected to 10–20% Tris-HCl gradient SDS-PAGE and electrotransferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Bedford, MA). scFvs on the membranes were detected using anti-flag M2-biotin-conjugated monoclonal antibody (Sigma) and then visualized by an enhanced chemiluminescence system (Amersham, Arlington Heights, IL) according to the manufacturers' protocol.

Determination of CD45 and LRP1 expression levels in the brain by immunoblot analysis

The supernatants of the neocortex homogenates were prepared as described above and mixed with $4 \times$ LDL sample buffer (Invitrogen) with and without dithiothreitol for CD45 and LRP1 detection, respectively. After heating at 100°C for 5 min, the samples (60 μ g/lane) were subjected to 7.5% SDS-PAGE and 8–16% gradient SDS-PAGE for CD45 and LRP1 detection, respectively. After electrotransfer to PVDF membranes (Millipore, Bedford, MA), low-density lipoprotein receptor-related protein 1 (LRP1) and CD45 were visualized using anti-LRP1-light chain (LC) (Calbiochem, No438192) and anti-CD45 (Abcam, ab10558) antibody, respectively, as described above. The membranes were reprobbed with anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody (Chemicon, Temecula, CA). The optical densities of protein bands from the membranes were determined by densitometric scanning using a HP Scanjet G3010 Photo Scanner and the Image J V1.40 (National Institutes of Health, USA). The optical density of each protein

band was divided by that of the GAPDH band on the same lane from the same membrane for normalization.

Statistical analysis

Data were expressed as mean $\pm$ SEM. Two-tailed Student's t-test was used to determine the intergroup difference. $P < 0.05$ was considered statistically significant.

Results

Transduction efficiency of rAAV serotype 1, 2, 6, and 8 in the skeletal muscle in adult C57BL/6 mice

Four serotypes of rAAV (1, 2, 6, and 8) encoding scFv59 were tested in adult C57BL/6 mice to determine the optimal rAAV serotype for intramuscular delivery of anti-A β scFv. The rAAV-CAscFv59 vectors [2.5×10^{11} vg/mouse] were injected into the right quadriceps muscle. Three months after injection, the mice were euthanized to evaluate the transduction efficiency of each rAAV serotype. The muscle sections were subjected to immunohistochemistry using anti-FLAG M2 antibody to visualize expressed scFv59 (scFv59 contains the Flag sequence as a marker). Among four serotypes of rAAV (1, 2, 6, and 8) encoding scFv59, rAAV1 had the highest levels of scFv59 expression (Fig. 1A). Although high levels of scFv59 expression were suggested by intensive immunostaining by anti-FLAG M2 antibody, inflammatory reactions were not discernible in the quadriceps muscles by histochemistry in any group. Therefore, rAAV1 was selected for testing the prophylactic efficacy of scFv59 in 3-month-old TgAPPswe/PS1dE9 mice. Nine months after rAAV1-CAscFv59 injection to the right quadriceps muscle, the mice were euthanized and levels of scFv59 expression were evaluated by immunohistochemistry and immunoblotting using anti-FLAG M2 antibody. As shown in Fig. 1E through G, strong expression of scFv59 was confirmed in the skeletal muscles of 12-month-old TgAPPswe/PS1dE9 mice even 9 months after rAAV-CAscFv59 injection. There was no decline of scFv59 expression (anti-FLAG M2 staining) in the skeletal muscles 9 months after rAAV-CAscFv59 injection compared to the scFv59 expression levels 3 months after the injection. Anti-FLAG M2 immunohistochemistry, however, failed to detect scFv59 in the brains of the TgAPPswe/PS1dE9 mice subjected to rAAV-CAscFv59 injection, suggesting that scFv59 did not reach A β deposits in the brains (data not shown).

rAAV1-mediated muscle-targeted scFv59 delivery reduces A β load in the brain

To determine the prophylactic efficacy of scFv59 expression in reducing A β load in the brain, diffuse and fibrillar A β deposits were detected by immunohistochemistry using anti-A β 6E10 antibody, and fibrillar A β deposits were visualized by thioflavin S fluorescence, followed by morphometric analysis (Fig. 2A through F). A β load was expressed as the percentage of area showing A β immunoreactivity or thioflavin S fluorescence. In the scFv59 group, the areas positive for 6E10 staining ($0.22 \pm 0.04\%$) and for thioflavin S fluorescence ($0.09 \pm 0.01\%$) in the hippocampus were significantly smaller than those (0.59 ± 0.08 and $0.18 \pm 0.01\%$, respectively, $P < 0.05$ for both) in the scFvGag group. In the neocortex, the thioflavin S fluorescence area of the scFv59 group ($0.11 \pm 0.02\%$) was smaller than that of the scFvGag group ($0.20 \pm 0.02\%$, $P = 0.015$). Although the 6E10-positive area of the scFv59 group in the neocortex ($0.42 \pm 0.05\%$) was smaller than that of the scFvGag group ($0.55 \pm 0.07\%$, $P = 0.16$), the difference was not significant, probably due to a large variation and/or small sample size.

rAAV1-mediated expression of scFv59 in the skeletal muscles reduced the hippocampal A β load in an AD mouse model. We determined if the decrease in the A β load in the hippocampus was associated with alterations in microglial activation using

immunohistochemistry. Activated microglia were visualized by the avidin-biotin immunoperoxidase method and anti-CD11b or Iba1 antibody. Microgliosis was quantified as the percentage of area showing immunoreactivity for CD11b or Iba1. Morphometric analysis of the immunostaining revealed no differences in immunoreactivity for CD11b and Iba1 between any groups (data not shown).

To evaluate further the prophylactic efficacy of rAAV1-mediated muscle-targeted scFv59 delivery, we determined both buffer-soluble and insoluble A β load by ELISA using A β 40 and A β 42 C-terminal-specific antibodies. No significant differences were found in levels of soluble A β 40 in the neocortex and hippocampus between the two groups (Fig. 3A). Levels of soluble A β 42 in the scFv59 group significantly reduced in the neocortex (0.53 ± 0.06 pg/mg protein) and hippocampus (0.13 ± 0.02 pg/mg protein) compared with the scFvGag group (0.90 ± 0.13 pg/mg protein, $P = 0.03$ and 0.39 ± 0.12 pg/mg protein, $P = 0.03$, respectively) (Fig. 3B). In the neocortex, levels of insoluble A β 40 (1.13 ± 0.26 ng/mg protein) and A β 42 (1.97 ± 0.36 ng/mg protein) in the scFv59 group reduced compared with the scFvGag group (2.22 ± 0.51 ng/mg protein, $P = 0.03$ and 3.80 ± 0.76 ng/mg protein, $P = 0.02$, respectively). In the hippocampus, however, no differences in levels of insoluble A β 40 and A β 42 were found between the two groups.

rAAV1-mediated muscle-targeted scFv59 delivery decreases levels of CSF A β

In order to investigate the possible mechanisms by which rAAV1-mediated muscle-targeted scFv59 delivery reduces cerebral A β load, we determined levels of A β 40 and A β 42 in CSF and blood. Levels of CSF A β 40 (4.91 ± 0.76 ng/ml) and A β 42 (1.35 ± 0.28 ng/ml) in the scFv59 group significantly reduced compared with those in the scFvGag groups (9.04 ± 0.91 ng/ml, $P = 0.01$ and 2.60 ± 0.27 ng/ml, $P = 0.01$, respectively) (Fig. 4). No difference was found in levels of serum A β 40 between the two groups (199 ± 39 pg/ml for the scFv59 group and 173 ± 109 pg/ml for the scFvGag group) (Fig. 5A). Although levels of serum A β 42 in the scFv59 group (28 ± 7 pg/ml) tended to increase compared to those in the scFvGag group (14 ± 4 pg/ml), the difference was not significant ($P = 0.20$), probably due to a large variation and/or small sample size (Fig. 5B).

To further investigate the possible mechanisms of reducing cerebral A β load by scFv59, LRP1 and CD45 in the neocortex were detected by immunoblotting using antibodies against LRP1-LC and CD45, respectively, (Fig. 6A) and their expression levels were compared between the scFv59 and scFvGag groups by densitometric analysis of the immunoblots (Fig. 6B). LRP1 is thought to mediate export of A β from the brain across the blood-brain barrier (Zlokovic 2004). CD45 is a leukocyte common antigen and protein-tyrosine phosphatase. Macrophages migrating into the brain from the periphery express greatly higher levels of CD45 than resident microglia (Guillemin and Brew 2004). Peripheral macrophages infiltrating into the brain are thought to more readily phagocytose and degrade A β than resident microglia (reviewed in Lai and McLaurin, 2012). There were no differences in steady-state levels of LRP1 and CD45 expression between the two groups (Fig. 6B).

Anti-scFv59 antibodies induced by rAAV1-mediated scFv59 expression in an AD mouse model are very modest and attenuate during aging

scFv59 was isolated by screening a human scFv library for A β immunoreactivity (Fukuchi et al. 2006a). scFv59 expression in mice may elicit immune responses. In order to investigate possible immune responses against scFv59 in mice, we determined levels of anti-scFv59 antibodies in sera, which may neutralize scFv59 binding activity to A β . Blood was collected at 2, 4, 6, and 8 months after rAAV injection and serum titers were quantified by ELISA. The levels of anti-59 antibodies were 5.9 ± 1.3 , 3.3 ± 1.25 , 2.0 ± 1.8 , and 0.6 ± 0.3 ng/ml at 2, 4, 6, and 8 months after rAAV injection (Fig. 6). The titers of anti-scFv59

antibodies were very modest and attenuated as the mice aged. Thus, their titers appeared to have insignificant effects on inactivating scFv59.

Discussion

Solomon et al. (1997) first indicated an immunotherapeutic approach for AD by showing that antibodies against A β 1–28 could disaggregate A β fibrils. Demonstration of the remarkable efficacy of A β immunization in abolishing A β deposits in an AD mouse model by Schenk et al. (1999) gave impetus to A β immunotherapy, leading to its clinical trials. The results from active (Check 2002; Orgogozo et al. 2003) and passive (Bard et al. 2000; DeMattos et al. 2001; Levites et al. 2006; Panza et al. 2010) immunization indicated that anti-A β immunotherapy could be a potentially powerful strategy to reduce A β deposits and improve cognitive deficits in patients with AD. Compared to active immunization, passive immunization may be preferred due to, at least, two reasons: 1) A small subset of AD patients subjected to an active immunization with A β vaccine (AN-1792) developed aseptic T-lymphocyte meningoencephalitis and/or Fc-mediated brain inflammation, which halted the clinical trial (Check 2002; Orgogozo et al. 2003), and 2) only 20% of AD patients developed adequate anti-A β antibodies in the AN-1792 trial presumably due to aging (immunosenescence) and low immunogenicity of A β (Sigurdsson et al. 2002). However, the chronic nature of AD requires long-term repeated administration of anti-A β antibodies, leading to a large financial burden. rAAV vectors encoding anti-A β scFvs are a potentially cost-effective alternative to passive immunization. It is possible to achieve long-term gene expression after a single administered dose of rAAV in many tissues including non-dividing cells like neurons and muscles (McCown 2005; Mingozzi and High 2011). AAV is nonpathogenic, nontoxic, and low immunogenic. scFvs consist of a single polypeptide chain, comprised of an antibody heavy chain variable domain (V_H) linked by a flexible polypeptide linker to a light chain variable domain (V_L) but exhibiting the same antigen binding properties as the parental complete monoclonal antibodies (Bird et al. 1988; Glockshuber et al. 1990; Huston et al. 1991). scFvs lack an Fc portion of immunoglobulin and cannot induce Fc-mediated inflammation. A variety of cell types and hosts can successfully express scFvs (Verma et al. 1998). We previously demonstrated that scFv59 brain delivery via rAAV was effective in reducing cerebral A β load in AD mouse models. However, the decrease in A β load was limited to the area where scFv59 was highly expressed in the brain (Fukuchi et al. 2006b; Kou et al. 2011). Therefore, we targeted the skeletal muscles to achieve scFv59 expression in the circulation in an attempt to reduce cerebral A β load globally. Additionally, the skeletal muscles are easily accessible and rich (Wang et al. 2005). First, we optimized scFv59 expression in the skeletal muscles by testing rAAV serotype 1, 2, 6, and 8 vectors. Widespread high expression of scFv59 was observed in the quadriceps muscle of rAAV1- and rAAV8-administered mice. Although immunohistochemistry revealed scFv59 expression in the whole quadriceps muscle when rAAV8 was injected, stronger immunostaining was achieved by rAAV1 in most of the quadriceps muscle compared to rAAV8 without observable inflammatory infiltration at the muscles (Fig. 1A through D). Therefore, rAAV1 was selected for preventive expression of scFv59 in an AD mouse model. Here, we have shown that AAV1-mediated muscle-targeted scFv59 gene delivery was effective in reducing cerebral A β load in an AD mouse model without causing meningoencephalitis and cerebral hemorrhages.

Wang et al. (2009; 2010) previously demonstrated that intramuscular delivery of anti-A β scFv via rAAV reduced cerebral A β deposits in an AD mouse model and improved their cognitive impairment without using an appropriate control. Additionally, they did not investigate A β levels in CSF and blood and the mechanism by which A β load was reduced. Because A β levels are in equilibrium among the brain, CSF, and blood, and appear to be tightly regulated in these organs (Shoji 2011), and because A β immunotherapy can reduce

cerebral A β load by altering the peripheral A β levels (DeMattos et al. 2001), we have determined A β levels in the brain, CSF, and blood in our experimental animals by ELISA. Furthermore, A β levels in the CSF and blood have been the subject of intensive investigation as potentially predictive biomarkers for disease progression and treatment response (Cummings 2011; Flood et al. 2011). In the current study, we injected rAAV1 vector encoding scFv59 directly into the quadriceps muscle of an AD model at 3 months of age. We have confirmed the widespread high expression of scFv59 in the skeletal muscles and showed that scFv59 muscle delivery was an effective modality in reducing cerebral A β deposits in an AD mouse model at 12 months of age. Because scFv59 was not found in the brain by immunohistochemistry, scFv59 may bind to A β in the periphery to enhance the A β efflux from the brain. However, we cannot exclude the possibility that scFv59 at indiscernible levels in the brain reacts with cerebral A β deposits to disaggregate and clear them from the brain. We did not find increases in activated microglia and monocyte/macrophage markers, CD11b, Iba1, and CD45, in the brain 9 months after the rAAV1 injection, suggesting that activated microglia/macrophages were not involved in A β clearance by scFv59. While scFv59 muscle delivery appeared to decrease A β levels in the brain and CSF, we found no changes in the levels of LRP1 expression, which mediates export of A β from the brain across the blood-brain barrier (Zlokovic 2004). However, it is possible that activated microglia/macrophage and/or LRP1 contributed to clearing brain A β at earlier stages of the current treatment. In summary, although the exact mechanism by which the cerebral A β load was reduced by the current modality is not clear, our data support the “peripheral sink” theory, which claims that anti-A β antibodies sequester peripheral A β and alter the equilibrium between the brain and blood, favoring the efflux of A β towards the periphery (DeMattos et al. 2001).

The reduction of A β in CSF may reflect its accumulation/deposition in the brains of patients with AD (Jack, Jr. et al. 2010; Perrin et al. 2009). Previous studies showed that levels of A β in the CSF and blood increased 24 h and 21 days after injection of anti-A β antibodies (DeMattos et al. 2001; Siemers et al. 2010). On the other hand, we found a reduction in CSF A β levels by muscular expression of scFv59 for 9 months. There are at least two chief differences between our experiments and those of others. We quantified A β levels after chronic treatment but the others did so after acute treatment. Subjects were prophylactically treated in our experiments but therapeutically in the others. Therefore, we infer that therapeutic treatment with anti-A β antibodies may acutely mobilize a large amount of A β from existing deposits into the CSF and blood, resulting in a remarkable increase in CSF and blood A β levels, but a longer treatment may reduce the A β mobilization capacity, leading to a reduction in CSF A β levels. Indeed, many of presumptive A β -modifying drugs including γ -secretase inhibitors, tramiprosate, and intravenous immunoglobulin decreased CSF A β levels after long-term treatment (Cummings 2011; Hampel et al. 2011). Thus, levels of A β in CSF appear to be important as a biomarker not only for AD diagnosis but also as a treatment response (Cummings 2011; Fagan et al. 2007; Hampel et al. 2011; Hansson et al. 2006).

Hippocampal A β deposits identified by immunohistochemistry and thioflavin S staining decreased in the scFv59 group compared with the scFvGag group (Fig. 2). On the contrary, no differences in levels of insoluble A β 40 and A β 42 by ELISA were found between the two groups (Fig. 3). Insoluble A β 40 and A β 42 were separately quantified by ELISA while these two major forms of A β deposits were measured together by immunohistochemistry and thioflavin staining. The differences in the A β deposits between the two treatment groups may be more readily discernible when A β 40 and A β 42 were measured together since both insoluble A β 40 and A β 42 levels in the scFv59 group tended to decrease compared with those in the scFvGag group.

One main concern of gene therapy is possible development of neutralizing antibodies against gene products, which are newly synthesized in animals. Recently, Chao and colleagues reported that muscle-directed coagulation factor IX (FIX) delivery via rAAV1 induced FIX-specific immune tolerance in mice and that induction of immune tolerance was dependent on the dose of rAAV1 and expression levels of FIX (higher the dose/expression, greater the tolerance) (Cohn et al. 2007; Kelly et al. 2009). In line with their observation, the induced anti-scFv59 antibody titers in mice subjected to muscular injection of rAAV1-CAscFv59 are very modest and the mice appeared to become tolerant to scFv59. Wang et al. (2010), however, found no neutralizing antibodies against their anti-A β human scFv in mice subjected to rAAV muscular injection. Although the reasons for this discrepancy are not clear, one possibility is the FLAG and histidine tag sequences. scFv59 has the tags but their scFv does not. Human scFv itself may not induce significant immune responses in mice. These results suggest that skeletal muscle can be a good target for scFv delivery via rAAV.

In conclusion, our results indicate that AAV1-mediated muscle-directed scFv59 gene delivery alters the equilibrium of A β between the brain and blood, facilitating the efflux of A β from the brain owing to antibody-mediated sequestration/clearance of peripheral A β and suggests that muscle-directed scFv59 delivery via rAAV1 may be a prophylactic option for AD.

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Fig. 1. scFv59 expression via rAAV serotype 1, 2, 6, and 8 in skeletal muscle. Optimization of scFv59 expression in the quadriceps muscle via rAAV serotype 1, 2, 6, and 8 (**A – D**). Four different serotypes of rAAV encoding scFv59 were injected into the quadriceps muscle of adult C57BL/6 mice. Three months after injection, scFv59 expressed in the muscle was visualized by immunohistochemistry using anti-FLAG M2 antibody. Widespread, strong expression of scFv59 is seen in the skeletal muscle by rAAV1-CAscFv59 injection (**A**). Very limited staining is observed by rAAV2-CAscFv59 (**B**). Partially strong expression of scFv59 is found after rAAV6-CAscFv59 injection (**C**). Widespread but weak expression of scFv59 in the muscle is observed by rAAV8-CAscFv59 (**D**). Scale bars, 500 μm (**A – D**). scFv59 expression in the skeletal muscle in TgAβPPswe/PS1dE9 mice nine months after PBS (**E**) or rAAV1-CAscFv59 (**F – H**) injection. PBS-injected muscle showing no staining by anti-FLAG M2 antibody (**E**). rAAV1-CAscFv59-injected muscle showing high expression of scFv59 (**F**). A high magnification (**G**) of the areas indicated by the squares in (**F**). Scale bar, 500 μm (**E** and **F**) and 150 μm (**G**). Nine months after rAAV1-CAscFv59 injection, muscle homogenates were prepared and subjected to 8–16% SDS-PAGE followed by immunoblotting using anti-FLAG M2 antibody. scFv59 is identified as an approximately 30kDa fragment in the muscle injected with rAAV1-CAscFv59 (**H**).

Fig. 2.

rAAV1-mediated scFv59 expression reduces Aβ deposits in the brain (A – F). Nine months after rAAV1-injection, TgAβPPswe/PS1dE9 mice were terminated and Aβ deposits in the brain were visualized and qualified by morphometric analysis. Aβ deposits are visualized by 6E10 antibody (A and B) and thioflavin S (D and E) in the brains after rAAV1-CAscFvGag (A and D) or rAAV1-CAscFv59 (B and E) treatment. The percentages of immunoreactive or fluorescent areas for 6E10 (C) and thioflavin S (F), respectively are shown as bar graphs. The values shown are the mean ± S.E.M. Scale bars 250 μm (A, B, D, and E).

Fig. 3. rAAV1-mediated scFv59 expression reduces Aβ levels in the brain. (**A** and **B**) The levels of soluble Aβ40 (**A**) and Aβ42 (**B**) in the neocortex and hippocampus are shown as the means ± S.E.M. pg/mg protein. The levels of insoluble Aβ40 (**C**) and Aβ42 (**D**) in the neocortex and hippocampus are shown as the means ± S.E.M. ng/mg protein (* P<0.05).

Fig. 4. rAAV1-mediated scFv59 expression reduces the levels of Aβ40 and Aβ42 in CSF. Aβ levels are shown as the means ± S.E.M. ng/ml (** P<0.01).

Fig. 5. rAAV1-mediated scFv59 expression has no effects on serum Aβ levels.

Fig. 6. rAAV1-mediated scFv59 expression does not alter steady-state levels of LRP1 and CD45 expression in the neocortices. Nine months after rAAV1-CAscFv59 injection, homogenates of the neocortices from the PBS, scFvGag, and scFv59 groups were subjected to SDS-PAGE followed by immunoblotting using anti-LRP1-LC, CD45, and GAPDH antibodies (A). The bar graph represents the ratios to the PBS group sample after normalizing the LRP1-LC and CD45 densities by the GAPDH densities (means $\pm$ SEM) (B).

Fig. 7.

Anti-scFv59 antibody titers induced by rAAV1-CAsFv59 injection are very modest and attenuate. 2, 4, 6, and 8 months after intramuscular injection of rAAV1-CAsFv59, sera were collected and anti-scFv59 IgG titers were determined by ELISA. The titers of anti-scFv59 antibodies in sera are shown as means $\pm$ S.E.M. ng/ml.