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Anti-A β single chain antibody delivery *via* adeno-associated virus for treatment of Alzheimer's disease

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Abstract

Immunization of mouse models of Alzheimer disease (AD) with amyloid-peptide (A β) reduces A β deposits and attenuates their memory and learning deficits. Recent clinical trials were halted due to meningoencephalitis, presumably induced by T cell mediated and/or Fc-mediated immune responses. Because injection of anti-A β F(ab')₂ antibodies also induces clearance of amyloid plaques in AD mouse models, we have tested a novel gene therapy modality where an adeno-associated virus (AAV) encoding anti-A β single chain antibody (scFv) is injected into the corticohippocampal regions of AD mouse models. One year after injection, expression of scFv was readily detectable in the neurons of the hippocampus without discernible neurotoxicity. AD mouse models subjected to AAV injection had much less amyloid deposits at the injection sites than the mouse models subjected to PBS injection. Because the scFv lacks the Fc portion of the immunoglobulin molecule, this modality may be a feasible solution for AD, without eliciting inflammation.

Keywords

gene therapy; vaccine; amyloid protein; amyloid plaque; transgenic mouse; immune therapy

1. Introduction

Alzheimer's disease (AD) is the most common cause of dementia after the age of 60. The pathological hallmarks of AD include deposition of amyloid β -peptide (A β) in neuritic plaques and cerebral blood vessels, neurofibrillary tangles, and loss of neurons. A β is derived from its

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precursor, APP, by proteolytic processing. Generation of A β requires cleavage of APP by β - and γ -secretase at the N-terminal and C-terminal end of A β , respectively. The amyloid cascade hypothesis claims that accumulation of A β in the brain is an initial and essential event in the pathogenesis of AD (Hardy and Selkoe, 2002). This hypothesis is supported by increasing lines of evidence: (1) Mutations in 3 different genes (APP, presenilin 1 and 2) implicated in the etiology of familial AD, have been shown to increase A β production, particularly a longer form of A β consisting of 42 amino acids (A β 1–42) (Scheuner et al., 1996); (2) A β 1–42 is considered to be highly amyloidogenic and oligomeric forms of A β are neurotoxic (Lambert et al., 2001; Walsh et al., 2002); (3) Patients with Down's syndrome (carrying three APP genes) develop AD pathology in their fourth or fifth decade of life; and (4) Overexpression of the mutant forms of APP in transgenic mice leads to AD-like pathologies including amyloid plaques in the brain. While the causes of sporadic AD are not clear, based on the similarities in the pathological and clinical manifestations between familial and sporadic AD, it is widely considered that A β also plays a pathogenetic role in sporadic AD.

The efficacy of A β vaccination on AD treatment was first demonstrated in a transgenic mouse model of AD, where immunization of AD mouse models with synthetic A β by repeated needle injection prevented or reduced A β deposits and improved their memory and learning deficits (Janus et al., 2000; Morgan et al., 2000; Schenk et al., 1999). These groundbreaking results prompted initiation of phase I/II clinical trials to evaluate the safety and tolerability of aggregated A β (AN1792) vaccination. AD patients subjected to this treatment showed less decline among the patients with positive antiserum titers against A β , compared with control patients in Disability Assessment for Dementia Scores, and no safety concerns arose during phase I clinical trial (Bayer et al., 2005). The phase IIa clinical trial, however, was halted due to meningoencephalitis found in 6 % of AD patients subjected to the vaccine trial (Orgogozo et al., 2003). Clinical studies and autopsy results indicated aseptic meningoencephalitis, presumably induced by T cell-mediated and/or Fc mediated immune responses (Ferrer et al., 2004; Nicoll et al., 2003; Orgogozo et al., 2003). In other experiments, peripheral administration of antibodies against A β induced clearance of preexisting amyloid plaques in an AD mouse model despite modest serum levels of the antibodies (Bard et al., 2000), indicating that an active T cell-mediated immune response was unnecessary. Based upon these observations, intravenous injection of humanized monoclonal antibodies against A β (passive immunization) has been proposed as a possible therapeutic means for AD and such a clinical trial has been started (Bacskai et al., 2001; DeMattos et al., 2001; Nitsch, 2004; Sigurdsson et al., 2002; Weksler, 2004). This modality, however, suffers from repeated administrations of antibodies, leading to a large financial and physical burden to AD patients. Because topical injection of Fc-removed antibodies against A β led to clearance of amyloid deposits in the AD mouse models (Bacskai et al., 2002), single chain antibodies (scFvs) against A β , which lack Fc, may be effective also. In this study, we attempt to demonstrate the feasibility of a new gene therapy modality for AD, where scFv against A β is delivered by adeno-associated virus (AAV) encapsidated in serotype 2 capsids to the brain. This approach may eliminate repeated administrations of antibodies or, as in the case for the clinical trial of gene therapy for hemophilia B (Kay et al., 2000; Manno et al., 2003), may reduce the number of antibody injections.

2. Materials and methods

2.1 Isolation of human scFvs against A β

A synthetic peptide containing amino acid residues 1–42 of A β was purchased from U.S. Peptide (Rancho Cucamonga, CA). scFvs that specifically react with the synthetic peptide were obtained through screening the ETH-2 human antibody phage library (Eidgenössische Technische Hochschule, Zurich, Swiss). The synthetic peptide was coated onto immunotubes

at a concentration of 20 µg/ml in PBS overnight at 4°C. Unbound antigen was washed away and the tubes were blocked by incubation with 2% dried milk in PBS for 2 h at room temperature. A library of approximately 10^{12} phages, encompassing 100–1000 copies of 10^9 distinct clones, was incubated with the immobilized antigen. After 2 h at room temperature, unbound phages were washed away and the remaining phages were eluted by exposure to 100 mM triethylamine. Logarithmically growing *E. coli* (suppressor strain TG1) was infected with a portion of the eluted phages, and the titers of eluted phages were determined by serial dilution. The remaining phages were grown in bacterial culture overnight, packaged and expressed through co-infection with helper phage, and precipitated from the bacterial supernatant. The precipitated phages were then used for subsequent rounds of antigen panning. Phages were isolated from single ampicillin-resistant colonies of infected TG1 cells using helper phage, and binding specificity for antigen was determined by enzyme-linked immunosorbent assay (ELISA). Ampicillin-resistant colonies were used to inoculate culture broth (200 µl/colony) in microtiter plates, and the expression of soluble scFv fragments was induced by addition of 1 mM isopropyl-D-thiogalactopyranoside to the cultures. Bacteria were pelleted, and the supernatants containing monoclonal phage populations were screened for binding to antigen by ELISA. Binding specificity was determined by comparing signals obtained from plates coated with the relevant antigen versus those obtained with the negative control antigen. Phages with high ELISA titer were isolated and used to infect the *E. coli* HB2151 (non-suppressor). Soluble scFv fragments induced by addition of 1 mM isopropyl-β-D-thiogalactopyranoside to the cultures were used to further screen scFv phage clones for binding to the synthetic Aβ1–42 peptide by ELISA using anti-FLAG M2 monoclonal antibody (scFv contains Flag sequences as a marker) (Sigma, St. Louis, MO) as the detecting reagent. An scFv clone, scFv59, that demonstrated the highest ELISA titer for Aβ immunoreactivity was selected for cloning of scFv cDNA. As a control, scFv-Gag was isolated by screening the ETH-2 library for HIV-1 Gag immunoreactivity.

2.2 Construction of an expression vector coding for scFvs

The cytomegalovirus promoter (CMV) in pAAV-MCS (Stratagene, La Jolla, CA) was replaced with CMV-enhancer/β-actin promoter (Niwa et al., 1991) to generate pAAV-CA. The woodchuck hepatitis virus post-transcriptional regulatory element (WPRE) was cloned by PCR using 5'-GGA AGC TTA ATC AAC CTC TGG ATT ACA A-3' and 5'-CTC TCG AGC AGG CCG GGA GGC GGC CCA A-3' as primers and pWHV8 as a template (ATCC, Manassas, VA) and inserted into the Hind III and Xho I sites of pAAV-CA to generate pAAV-CAWPRE. The Kozak and signal sequences of the immunoglobulin heavy chain (VH) gene were prepared by annealing and ligating 4 overlapping oligonucleotides listed in Table 1. The ligated oligonucleotides contained EcoR I and BsrG I restriction enzyme sites (bold letters in the table) at the 5' and 3' ends of the sequences, respectively. cDNA for scFv was isolated by PCR using 2 primers (5'-TGG **TGT ACA** GTG TGA GGT GCA GCT GTT GGA GTC TGG G-3' 5'-CCA **AGC TTC** TAA TGG TGA TGG TGA TGG TGC TTG TCG TC-3') with Nco I and Hind III restriction enzyme sites (in bold letters). After adding the signal sequences of the VH gene to the 5' of the PCR product in frame, the signal and scFv cDNA was inserted into the Eco RI and Hind III sites of pAAV-CAWPRE to generate pAAV-CAscFv59 (Fig. 1). pAAV-CAscFv-Gag was similarly prepared by cloning cDNA for scFv-Gag into pAAV-CAWPRE. In the vectors, produced scFvs contained the D3SD2-FLAG-His tag as a marker at the C-terminal ends. The DNA fragments prepared by PCR were verified by DNA sequencing.

2.3 Expression and secretion of scFv from cultured cells

COS (an African green monkey kidney cell line) cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 % fetal bovine serum in a 60 mm dish and transfected with 10 µg of pAAV-CAscFv59 or pAAV-CAscFv-Gag using metafectene (Biontex, Munich, Germany) according to the manufacturer's protocol. Seventy-two hours

after transfection, the media and cells were harvested and the proteins in the media and cells were separated by 4–15% SDS-PAGE. After electrotransfer to polyvinylidene difluoride (PVDF) membranes (Immobilon-P, Millipore, Bedford, MA), scFvs were visualized by anti-FLAG M2 antibody (Sigma) using the avidin-biotin immunoperoxidase method (Vectastain C kit, Vector, Burlingame, CA) followed by the enhanced chemiluminescence method (Amersham, Arlington Heights, IL) according to the manufacturers' protocols.

2.4 Immunoreactivity of secreted scFv to amyloid deposits in plaques and blood vessels

To assess amyloid plaque-immunoreactivity of secreted scFv from COS cells transfected with pAAV-CAscFv59, sections of formalin fixed, paraffin embedded brain from a patient with AD were subjected to the immunoperoxidase method. The brain sections were obtained from the University of Alabama at Birmingham Alzheimer's Disease Center. Endogenous peroxidase was eliminated by treatment with 3% H₂O₂ for 30 min after deparaffinization of the sections. After washing with distilled water, the sections were treated with 88% formic acid and rinsed with water and 0.1 M Tris-buffered saline (TBS) (pH 7.4). The sections were blocked with 15% horse serum in TBS for 60 min at room temperature and incubated with a diluted medium [1:4 with 5% horse serum in 0.1 M Tris-saline (pH 7.4)] from the transfected COS cells as a primary antibody for 16 hrs at 4°C. The sections were rinsed in 0.1 M TBS containing 1% serum and incubated with an anti-Flag M2 antibody (secondary antibody) for 60 min at room temperature. After washing with 0.1 M TBS containing 1% serum, the sections were incubated with biotinylated anti-mouse IgG antibody (tertiary antibody) for 60 min at room temperature. After washing, the sections were incubated with Vectastain ABC reagent (Vector, Burlingame, CA) for 60 min at room temperature. Peroxidase activity was detected by treatment with 3,3'-diaminobenzidine. The sections were counterstained with hematoxylin. A medium from COS cells transfected with pAAV-CAscFv-Gag was used as a control. 6E10 (a mouse monoclonal antibody raised against amino acid residues 1–16 of A β , Signet, Dedham, Massachusetts) was used for comparison, also.

2.5 Production of rAAV

rAAV was prepared using the iodixanol gradient method as described by (Zolotukhin et al., 2002) with a modification. HEK293 cells were cultured in DMEM supplemented with 10 % fetal bovine serum. In brief, approximately 3×10^6 HEK293 cells per 100-mm tissue culture dish were inoculated for 48 h prior to transfection. Seventy to eighty percent confluent HEK293 cells were transfected with 8 μ g each of pAAV-CAscFv59, pAAV-RC (Stratagene, La Jolla, CA) and pHelper (Stratagene), using metafectene according to the manufacturer's protocol. Cells were incubated at 37 °C, 5% CO₂ for 60 h and harvested using PBS containing 5 mM EDTA. The collected cells were centrifuged at 1,000g for 10 min, resuspended in lysis solution (150 mM NaCl, 50 mM Tris, pH 8.4) and lysed by three freeze/thaw cycles between dry ice-ethanol and 37 °C water baths. Benzonase (Sigma, St. Louis, MO) was then added to the cell lysate (50 U/ml final concentration) and incubated for 30 min at 37 °C. The crude lysate was centrifuged at 4,000g for 20 min and the vector-containing supernatant was divided between four iodixanol gradients. Each gradient consisted of (from the bottom) 5 ml 60%, 5 ml 40%, 6 ml 25%, and 9 ml of 15% iodixanol; the 15% density step also contains 1 M NaCl in a quick-seal tube (25×89 mm, Beckman) with 15 ml cell lysate at the top. Iodixanol was prepared using a 60% (w/v) sterile solution of OptiPrep (Nycomed) and PBS-MK buffer (1× PBS containing 1 mM MgCl₂ and 2.5 mM KCl). Tubes were centrifuged in a Type 70 Ti rotor at 69,000 rpm (350,000g) for 1 h at 18 °C. Approximately 5 ml of the 40–60% step interface were aspirated after side-puncturing each tube with a syringe equipped with an 18-gauge needle. The iodixanol gradient fraction was further purified by a 3-ml heparin-agarose Type I column (Sigma). The column was equilibrated with 10 ml of PBS-MK buffer, then 10 ml of PBS-MK/1 M NaCl, followed by 20 ml of PBS-MK buffer. The vector-containing iodixanol fraction (20 ml) was loaded onto the column by gravity flow. The column was washed with 20 ml of PBS-MK buffer

and eluted in 15 ml of PBS-MK/1 M NaCl. The vector was concentrated and buffer-exchanged with PBS using a Amicon Ultra 100 K concentrator (Millipore, Bedford, MA).

2.6 Dot-blot assay for rAAV physical particle titer

The dot-blot assay was used to determine the titer of rAAV virions that contained vector genomes. After digesting plasmid and unpackaged vector with DNase I, encapsidated rAAV vector genomes were liberated by proteinase K treatment. The liberated vector DNA was phenol extracted and ethanol precipitated using glycogen as a carrier. Precipitated DNA was dissolved in distilled H₂O and diluted into 400 μ l 0.4 N NaOH/10 mM EDTA at various concentrations. Denatured vector DNA was immobilized onto a nylon membrane along with the known concentrations of pAAV-CAscFv59 using a dot-blot apparatus (Bio-Rad, Hercules, CA). The blots were probed with labeled scFv cDNA using North2South direct HRP labeling and detection kit (Pierce, Rockford, IL) according to the manufacturer's protocol. The vector DNA signal was compared with the signal generated from the plasmid DNA standard curve, and extrapolated to determine a vector genome titer.

2.7 Stereotaxic injection of rAAV-CAscFv59 to the hippocampus and neocortex of Tg2576 mice

Five Tg2576 mice at the age of 15 months were subjected to rAAV-CAscFv59 injection and, as controls, five age- and sex-matched Tg2576 mice were injected with PBS. Tg2576 mice used here were kept on the C57BL/6 background as previously described (Kim et al., 2004; Li et al., 2003). All animal procedures used for this study were prospectively reviewed and approved by the Institutional Animal Care and Use Committees of the University of Alabama at Birmingham and the University of Illinois College of Medicine at Peoria. Mice were anesthetized by pentobarbital and placed on a stereotaxic instrument with a motorized stereotaxic injector (Stoelting, Wood Dale, IL). A hole on the skull was made by a drill 0.2cm posterior to the bregma and 0.15 cm to the midline for the corticohippocampal injection. Two bilateral injections (2 μ l each) were performed at the depth of 0.17 (hippocampus) and 0.07 (neocortex) cm at a rate of 1 μ l/min. After allowing the needle to remain in place for 5 min, the needle was slowly raised at a rate of 0.1 cm/min. A total of 8 μ l [2×10^9 genomes (vg)]/mouse was injected.

2.8 Amyloid load by immunohistochemistry

The immunohistochemical method has been described previously in (Li et al., 2003). The mice were deeply anesthetized by intraperitoneal injection of sodium pentobarbital and the brains were quickly removed. The brains were fixed in 10% formaldehyde: 90% alcohol and embedded in paraffin. For the hippocampus and neocortex (coronal sections at the injection site spreading 1.2 mm on a rostro-caudal axis), five sections, each separated by consecutive 120 μ m intervals, were analyzed. Three micron tissue sections were subjected to the avidin-biotin immunoperoxidase method using 6E10 antibody and Vectastain ABC kit. The amyloid burden in the neocortex and hippocampus of mouse brain was quantified by histomorphometry, consisting of a Leica DMR research microscope with a SPOT RT Slider digital camera (Diagnostic Instruments, Sterling Heights, Michigan) and the Image Pro Plus v4 image analysis software (Media Cybernetics, Silver Spring, Maryland) capable of color segmentation and automation via programmable macros. The entire hippocampus and neocortex within 3 mm from the longitudinal fissure in each slide were scanned. Thirty to forty fields (1 mm² each, using a 10 $\times$ objective and a 1 $\times$ eyepiece lens) from 5 coronal brain sections from each mouse at the injection site were analyzed. Amyloid burden was expressed as a percentage of total area covered by A β immunoreactivity.

2.9 Histopathological analysis

Brain sections of mice subjected to rAAV-CAscFv59 or PBS injection were prepared as in the immunohistochemistry method above (5 sections for each mouse). The brain sections were stained with H & E (hematoxylin-eosin) for general pathology. Possible cerebral hemorrhage in the experimental mice was investigated by iron stain (Prussian blue reaction) using a Sigma-Aldrich Iron Stain kit (Sigma-Aldrich, St. Louis, MO) according to the manufacturer's protocol. For each mouse, 5 coronal brain sections (approximately 60 μm^2 of neocortex and hippocampus for each mouse) separated by consecutive $\sim 120 \mu\text{m}$ at the injection site were analyzed by iron stain ($n = 5$ for each group).

2.10 Long-term expression of scFv and cytotoxicity assay

To evaluate long term expression of transgene and possible toxicity of long-term expression of scFv in the brain, rAAV-CAscFv59 [5×10^9 vector genomes (vg)/hemisphere] was injected into the hippocampal regions of 4-month-old C57BL6 mice as described above. Three weeks and one year after injection, the mice were terminated and subjected to immunohistochemistry using avidin-biotin immunoperoxidase method and anti-FLAG M2 antibody as described above. To investigate possible toxicity of scFv59, the brain sections were subjected to Terminal (TdT)-mediated dUTP-biotin nick end labeling (TUNEL) assay using the In situ Cell Death Detection Kit (Roche Biochemicals, Indianapolis, IN) according to the manufacturer's protocol. Slides were analyzed under a fluorescence microscope (OLYMPUS IX71).

2.11 Statistical analysis

Data were expressed as mean $\pm$ standard error. Inter group differences were assessed by two-tailed Student's t-test and two-way analysis of variance (ANOVA) using Statview software. $P < 0.05$ was considered statistically significant.

3. Results

3.1 scFv is secreted from COS cells after pAAV-CAscFv59 transfection and secreted scFv reacts with amyloid deposits in neuritic plaques and blood vessels

To confirm secretion of scFv59 from cells, an expression vector, pAAV-CAscFv59, was introduced into COS cells and, 72 h later, 20 μg of COS cell lysate and 10 μl of the conditioned medium were separated by 4–15% SDS-PAGE, and western blot analysis was performed. scFv59 was detected as a 35 kDa fragment by anti-FLAG M2 antibody (scFv contain the FLAG sequence as a marker) in cell lysates and medium from COS cells (Fig. 2A). Thus, scFv59 was expressed in cells and secreted into the media. Secretion of scFv-Gag from COS cells was similarly confirmed after transfection with pAAV-CAscFv-Gag (data not shown). Immunohistochemical analysis was carried out to assess anti-A β immunoreactivity of scFv59 secreted from COS cells. When a brain section of an AD patient was stained with the medium from COS cells transfected with pAAV-CAscFv59, as a source of primary antibody, and anti-FLAG M2 antibody as a secondary antibody by the avidin-biotin immunoperoxidase method, neuritic and diffuse plaques in the brain section were visualized (Fig. 2B). Vascular amyloid was also stained with the medium containing scFv59 (Fig. 2B). The staining pattern of scFv59 was similar to that of 6E10 antibody, except that more amyloid deposits with less than $\sim 20 \mu\text{m}$ diameters were visualized by 6E10 antibody as compared with scFv59 (Fig. 2B and C). No amyloid deposits were detected by a control medium from COS cells transfected with pAAV-CAscFv-Gag (Fig. 2D). These results indicate that scFv59 secreted from COS cells is immunoreactive with most of amyloid deposits detected by 6E10 antibody.

3.2 Effects of scFv59 brain expression via adeno-associated virus on amyloid deposition in Tg2576 mice

Tg2576 mice were terminated in order to evaluate the efficacy of rAAV-CAscFv59 injection in clearing A β deposits at the injection sites. The amyloid load at the injection sites (the hippocampus and neocortex at the injection site spreading 1.2 mm on a rostro-caudal axis and within 3 mm from the midline) was determined by morphometric analysis of immunohistochemically stained brain sections from the Tg2576 mice using anti-A β 6E10 antibody. Representative results are shown in Fig. 3A and B. The β -amyloid loads (average percentage of area showing A β immunoreactivity) in the hippocampus and neocortex were on average $3.09 \pm 0.718\%$ and $5.97 \pm 1.08\%$ for rAAV-CAscFv59 injected and PBS injected Tg2576 mice, respectively ($P < 0.05$) (Fig. 3C). Thus, Tg2576 mice subjected to rAAV-CAscFv59 injection had fewer A β deposits than Tg2576 mice injected with PBS.

3.3 Effects of scFv59 brain expression via adeno-associated virus on cerebral hemorrhage in Tg2576 mice

Possible cerebral hemorrhage in all the experimental mice was investigated by iron stain (Prussian blue reaction). For each mouse, 5 coronal brain sections separated by consecutive $\sim 120\ \mu\text{m}$ at the injection site were examined. Each brain section contained hippocampus and neocortex. One of the Tg2576 mice injected with PBS showed scattered stains along with the needle track, which appear to be associated with phagocytic cells (Fig. 4A and B). Several phagocytic cells showing blue staining were found in two other Tg2576 mice injected with PBS and in one Tg2576 mouse injected with rAAV-CAscFv59. The others did not show hemorrhage (Fig. 4C). No hemorrhage associated with blood vessels was found in any of the mice. Thus, Tg2576 mice subjected to rAAV-CAscFv59 injection did not show an increase in cerebral hemorrhage as compared with the controls. Histopathological examination (H & E staining) revealed no evidence of significant neuronal necrosis or demyelination in Tg2576 mice subjected to rAAV-CAscFv59 injection compared with Tg2576 mice injected with PBS.

3.4 Effects of long-term expression of scFv59 on cell viability

We studied possible cytotoxicity of long-term expression of scFv in brain using non-transgenic mice because A β is thought to be neurotoxic. To study scFv expression in neural cells, rAAV-CAscFv59 (5×10^9 vg/hemisphere) was injected into the hippocampus of C57BL/6 mice. Three weeks after injection, the mice were terminated and subjected to immunohistochemistry using avidin-biotin peroxidase method and anti-FLAG M2 antibody against scFv. The granule cell layers of the dentate gyrus and CA3 pyramidal neurons were heavily stained, suggesting accumulation of scFv within the cytoplasm and dendrites (Fig. 5A). The neuropil of the dentate gyrus and hippocampus CA3 was also strongly stained, suggesting secretion of scFv from neurons. To evaluate long term expression of scFv and its cytotoxicity in the brain, rAAV-CAscFv59 (5×10^9 vg/hemisphere) was injected into the hippocampus of 4-month old mice. One year after the injection, the mice (16 months old) were sacrificed for immunohistochemical detection of scFv59 and TUNEL. Some pyramidal neurons in the hippocampus were stained with anti-FLAG M2 antibody (Fig. 5C), suggesting a sustained expression of scFv in neurons for one year. No cell death was detected by TUNEL assay (Fig. 5E) and no difference was seen between rAAV-CAscFv59- and PBS-injected mice, suggesting that scFv is not toxic to cells, even after sustained expression of scFv for one year.

4. Discussion

In attempting to develop safe, effective immune therapy for AD, we have demonstrated the feasibility of a novel gene therapy modality, where anti-A β antibody is delivered to the brain by rAAV. Six months after corticohippocampal injection of rAAV-CAscFv59, less amyloid deposits were found at the injection sites in Tg2576 mice compared with Tg2576 mice

subjected to PBS injection. Long-term expression of scFv59 in the hippocampus without discernible cytotoxicity was confirmed by histochemical and immunohistochemical analyses and TUNEL assay, suggesting that this gene therapy modality may be a safe, effective treatment for AD.

Stability is an essential property for proteins with biomedical activities. Some scFvs may have limited stability compared with complete antibodies (Brockmann et al., 2005; Worn and Pluckthun, 2001). To study stability and function of secreted scFv59 from cells, we have transfected pAAV-CAscFv59 into COS cells and performed western blot and immunohistochemical analyses. scFv59 secreted from COS cells was immunoreactive with plaque and vascular amyloid (Fig. 2B). No degradation derivatives from scFv59 were detected by western blot analysis using anti-FLAG M2 antibody. The intensity of the band for scFv59 in the conditioned medium (10 μ l/lane) by western blotting was comparable to that in the COS cell lysate (20 μ g/lane) (Fig. 2A). The COS cells were cultured in a 60 mm-dish with 5 ml of the medium and yielded approximately 1.2 mg of total protein. Thus, the amount of scFv59 in the medium was approximately 8 times higher than that in the cell lysate. These results suggest that secreted scFv59 is stable and functional as an antibody.

AAV is a single-stranded DNA human parvovirus with a 4.7 kb genome. Among the many viruses currently being developed as vectors for human gene therapy, AAV is one of the most promising tools because AAV is nonpathogenic, nontoxic with low immunogenicity, and allows long-term gene expression in many tissues, including nondividing cells like neurons. rAAVs have been successfully used in a number of animal models of neurological disorders (Burger et al., 2005; McCown, 2005; Verma and Weitzman, 2005). rAAV2 is the most commonly used serotype and transduces both neurons and ependymal cells in the central nervous system (Davidson et al., 2000). WPRE is effective in enhancing transgene expression by rAAV in brain (Klein et al., 2002; Paterna et al., 2000). Spatial and temporal expression of transgenes by rAAV2 may be influenced by promoters and brain areas. scFv59 expression in the hippocampus is mostly limited to the hilar region, which is consistent with the reports by Klugmann et al. (2005) and Burger et al. (2004). This spatial limitation can be overcome by the use of other serotypes, such as AAV1, AAV5, and AAV8 (Burger et al., 2004; Di Pasquale et al., 2003; Klein et al., 2005; Klugmann et al., 2005; Richichi et al., 2004). The CMV promoter has been commonly used to drive transgene expression in the brain and gives varying levels of expression throughout the brain but mostly in neurons (~70% of transduced cells) (Ehrensgruber et al., 2001). The levels of transgene expression by the CMV promoter may diminish with time in some brain areas (McCown et al., 1996). Although scFv59 is readily detectable in the hippocampus one year after injection, the number of positive cells for scFv59 staining seems to decrease compared with the levels seen 3 weeks after injection (Fig. 5A and C). The reasons for this decline in scFv expression are not clear but may be associated with methylation because the CBA promoter contains CMV sequences (Lo et al., 1999; Prosch et al., 1996).

Passive immunization treatment with anti-A β antibody is thought to be a therapeutic option for AD. Bard et al. (2003) reported that, among anti-A β antibodies tested for passive immunization, only antibodies against N-terminal epitope of A β cleared amyloid plaques in PDAPP transgenic mice, an AD mouse model, and that A β plaque binding correlated with a clearance response and neuronal protection. Pfeifer et al. (2002) however, reported that passive immunization with anti-A β antibodies directed against N-terminal epitopes of A β increased cerebral microhemorrhage in APP23 mice, another mouse model of AD, which developed significant deposits of A β in the cerebral vasculature [cerebral amyloid angiopathy (CAA)] (Calhoun et al., 1999). One of AD patients subjected to the clinical trial of AN1792 had multiple cortical hemorrhages (Ferrer et al. 2004), also. Racke et al. (2005) reported that passive immunization of anti-A β antibodies immunoreactive with deposited A β in parenchyma and

vessels caused microhemorrhage in PDAPP transgenic mice. Microhemorrhage caused by anti-A β antibody administration in Tg2576 mice older than 19 months of age was associated with an increase in vascular A β deposits (Wilcock et al., 2003). scFv59 is immunoreactive with deposited A β in brain (Fig. 2B). Therefore, we performed Prussian blue staining on experimental mice. We did not find an increase in iron stain in Tg2576 mice subjected to rAAV-CAscFv59 injection compared to control subjects. No hemorrhage associated with blood vessels was found. The reasons for this discrepancy are not clear. scFv consists of a single polypeptide chain, comprised of an antibody heavy chain variable domain linked by a flexible polypeptide linker to a light chain variable domain. The structural difference between scFv and a whole antibody can be one of the reasons. Thus, scFvs against A β may be a safer option for AD treatment. Alternatively, widespread expression of scFv59 may cause hemorrhage because expression of scFv59 appeared to be limited to the vicinity of the injection site in our experiments.

Neurotoxicity of A β was first demonstrated by Yankner and his colleagues and the fibrillar form of A β was thought to be toxic (Yankner et al., 1990; Yankner, 1996). However, more recent experiments demonstrated that soluble A β oligomers were more toxic than A β fibrils (Dahlgren et al., 2002; Klein et al., 2002; Lambert et al., 1998; Walsh et al., 2002). In accordance with the latter observations, AD mouse models showed altered synaptic morphology and electrophysiological changes before A β deposits (A β fibrils) developed in the brain (Hsia et al., 1999; Mucke et al., 2000). Because A β oligomers were produced within cells and shed from the cells (Walsh et al., 2002), several investigators proposed reducing intracellular and extracellular A β oligomers as an efficient therapeutic target (Bayer et al., 2001; Lambert et al., 2001; Walsh et al., 2002). Based upon this hypothesis, Paganetti et al. (2005) demonstrated that intracellular expression of scFv against the β -secretase cleavage site of APP is effective in preventing production of A β from APP. scFv59 is immunoreactive with oligomeric A β , and inhibits A β aggregation and provably cytotoxicity *in vitro* (Fukuchi et al., 2006). Such properties of scFv59 are concordant with those of other anti-A β scFvs characterized by some investigators (Liu et al., 2004; Manoutcharian et al., 2004; Solomon and Frenkel, 2002). Intracellular expression of anti-A β scFv via rAAV in neurons may be more effective in treating AD patients than passive and active immunization. Our preliminary data suggests that corticohippocampal injection of rAAV-CAscFv59 may be effective in improving learning and memory deficits in Tg2576 mice.

In summary, we have demonstrated the feasibility of a gene therapy modality for AD, in which anti-A β antibodies have been delivered to the brain by rAAV encoding the antibodies. This modality for AD should be further exploited to develop safe and effective treatment for AD.

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Fig. 1.

Construction of pAAV-CA-scFv59 vector. ITR: inverted terminal repeats; CMV: cytomegalovirus enhancer; β -actin: β -actin promoter; SS: signal sequence; scFv: cDNA for single chain antibody 59; WPRE: woodchuck hepatitis virus post-transcriptional regulatory element; hGH polyA: human growth hormone polyadenylation signal.

Fig. 2.

Secretion and A β -immunoreactivity of scFv59. (A) Expression and secretion of scFv59 in cultured COS cells. COS cells were transfected with pAAV-CAscFv59, or pAAV-CAWP (no cDNA insert). Three days after transfection, the media were collected and cells were harvested. Twenty ng protein/lane of cell lysate was loaded on 4–15% Tris-HCl-polyacrylamide gel. For media, 10nl of medium from each sample was mixed with 10 μ l of 2 $\times$ Laemmli buffer and loaded on the same gel. After electrophoresis and electroblotting to a PVDF membrane, scFv59 was visualized by anti-FLAG M2 antibody. (B) scFv59 secreted from COS cells reacts with A β deposits in blood vessels as well as in neuritic and diffuse plaques in a brain section from a patient with AD. (C) A serial section of (B) stained with 6E10 anti-A β antibody for comparison. (D) A serial section of (B) immunostained with scFv-Gag secreted from COS cells shows no staining. COS cells were transfected with pAAV-CAscFv59 or pAAV-CAscFv-Gag and, 3 days after transfection, media were harvested. The media were diluted to 1:4 with 5% horse serum in 0.1 M Tris-saline (pH 7.4) and used as primary antibodies. For detection of scFvs, the mouse monoclonal anti-FLAG M2 antibody and biotinylated anti-mouse IgG were used as secondary and tertiary antibody, respectively. Biotinylated anti-mouse IgG was used as secondary antibody for 6E10 antibody. The arrowheads, arrows, and asterisks indicate A β deposits in blood vessels, neuritic, and diffuse plaques, respectively. Scale bars 200 μ m (B through D).

Fig. 3. rAAV-CAscFv59 injection reduces Aβ deposits in the brain of Tg2576 mice. Aβ deposits were detected with 6E10 antibody. (A) rAAV-CAscFv59-injected brain. (B) PBS-injected brain. (C) Morphometric analysis of Aβ load. Average percentages of area showing Aβ immunoreactivity are indicated (n = 5 for each group). Scale bars 250 μm (A and B).

Fig 4. Prussian blue staining for detection of hemorrhages in Tg2576 mice. (A) and (B) Blue staining spots scattered through the needle track are seen in the boxed area of the hippocampus of a PBS-injected Tg2576 mouse. (B) is a higher magnification of the boxed area in (A). In order to show iron staining clearly, the picture was taken before counter staining with pararosaniline. Arrows indicate iron staining associated with phagocytic cells. (C) A brain section of a Tg2576 mouse subjected to rAAV-CAscFv59 injection shows no staining. Scale bars 50 μ m (A and C) and 10 μ m (B).

Fig 5.

scFv59 is readily detectable in the brain three weeks and one year after injection of rAAV-CAscFv59 without showing cytotoxicity. (A) rAAV-CAscFv59 (5×10^9 vg/hemisphere) was injected into the hippocampal regions of C57BL/6 mice. Three weeks later, expressed scFv59 was detected in the hippocampus using anti-FLAG M2 antibody.

The arrowheads indicate heavy neuropil staining in the dentate gyrus. (B) As a control, PBS-injected mouse brain is shown. (C) rAAV-CAscFv59 was similarly injected into the hippocampal regions of mice. One year after injection, expression of scFv59 was similarly detected. (D) As a control, PBS-injected mouse brain is shown. (E) Possible cell death (apoptosis) associated with long-term expression of scFv59 in the brain of the mice was investigated by TUNEL staining. (F) As a control, PBS-injected mouse brain was used. The insets are higher magnifications of the boxed area indicated by the arrows. Scale bars 200 μ m (A through F) and 40 μ m (insets).

Table 1
Oligonucleotides for the Kozak and signal sequences of the immunoglobulin heavy chain (VH) gene

DNA sequences of oligonucleotides

5'-CGG **AAT TC**¹*G CCA CC*² A TGG AGT TTG GGC TGA GCT-3'
5'-GGC TTT T³TC TTG TGG CTA TTT TAA AAG GTG **GTG TAC A**⁴ GT G-3'
5'-CAC **TGT ACA**⁴ CCA CCT TTT AAA ATA GCC ACA AGA-3'
5'-AAA AGC C³AG CTC AGC CCA AAC TCC ATG *GTG GCG AAT TC*¹ C G-3'

¹ EcoR I site is in bold.

² Kozak sequence is in italics.

³ Overlapping sequences are underlined.

⁴ BsrG I site is in bold.