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ADENO-ASSOCIATED VIRAL VECTOR-MEDIATED APOE EXPRESSION IN ALZHEIMER'S DISEASE MICE: LOW CNS IMMUNE RESPONSE, LONG-TERM EXPRESSION, AND ASTROCYTE SPECIFICITY

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1. ABSTRACT

Recombinant Adenovirus and Adeno-associated virus (AAV) are highly effective vehicles for gene transfer into CNS cells. However, the duration of gene expression and the cytotoxicity to cells are quite different between these viral approaches. We initially investigated these distinctions by stereotaxically injecting both Adenovirus vector and AAV vectors expressing reporter genes into mouse hippocampus. The adenovirus vector induced a pronounced immune response with a marked increase in CD45 and MHC class I protein expression and transgene expression was shorter than six weeks. In contrast, with the AAV vector there was lower expression of CD45 and MHC class I immune activation markers, and longer expression of reporter gene (up to 12 months). To study the roles of human Apolipoprotein E (ApoE) alleles in the pathogenesis of Alzheimer's disease and other CNS diseases, we generated recombinant AAV-apoE alleles driven by the GFAP promoter and expressed them in the mouse brain of Alzheimer's disease mouse. High level ApoE expressions in mouse brain lasted for 12 months, and ApoE was specifically expressed in astrocytes. We demonstrate that AAV-GFAP-ApoE is valuable in studying the pathogenesis and in gene therapy for Alzheimer's disease and other CNS diseases.

2. INTRODUCTION

The Adenovirus viral vector has been widely used as an effective vehicle for gene delivery to the CNS in animal models. It can carry large pieces of foreign genes (up to 8 kb), is generated at high titer, and infects both dividing and non-dividing cells. The transferred DNA remains as a non-integrated episome in the nucleus and is therefore sufficient for transient expression of transgenes in non-dividing cells. In comparison, adeno-associated virus (AAV) is not pathogenic to humans. 96% of the viral genome is removed from the AAV vector, leaving only the two short inverted-terminal repeats (ITRs) that are sufficient for viral packaging and integration. The AAV vector can therefore integrate into host chromosomes and stably express transgenes in both dividing and non-dividing cells. It triggers minimal immune responses yet maintains transgene expression for a long time (1). In addition, recently designed rAAV vectors are able to produce much higher infectious particles, and as well are able to use Adenovirus-free preparations (2, 12, 4). AAV has distinct advantages because it is less immunogenic, less cytotoxic, and has long-term expression compared to other viruses (e.g., HSV) used for CNS gene delivery, and is a valuable tool for CNS gene therapy.

Apolipoprotein E (ApoE) is a plasma protein that plays an essential role in the transportation of cholesterol and phospholipids. Human ApoE exists in 3 isoforms (E2, E3, and E4) that differ by single amino acids at position 112 (cysteine *vs.* arginine) and/or 158 (cysteine *vs.* arginine). The most common allele is E3 (77%), next E4 (15%), and finally, E2 (8%) (5). Recent studies have suggested roles for ApoE isoforms in some of the primary pathological processes of Alzheimer's disease (AD). ApoE isoforms are detected in the two characteristic neuropathological lesions of AD (neuritic amyloid plaques and intracellular neurofibrillary tangles). In AD, there is a significant correlation between early age of onset and presence of the ApoE4 allele (6). ApoE4 is associated with either accelerated cognitive aging or subclinical dementia, whereas the ApoE2 allele is associated with decreased risk for AD and delayed AD onset. At least some elderly

ApoE4 subjects who perform more poorly than their non-E4 counterparts are more likely to progress to a clinical diagnosis of dementia after 3-4 years (7, 8, 9, 10).
To better understand the biological and pathological functions of ApoE isoforms in AD, we initially used recombinant Adenovirus and AAV mediated gene delivery to express ApoE alleles in the CNS of normal aged mice and Alzheimer's disease (AD) mice. We compared the time course of expression, immune activity, and cell type specificity of these viral vectors. We further generated recombinant AAV-GFAP-ApoE and specifically expressed ApoE in astrocytes of the AD mouse hippocampus. We demonstrated that AAV-GFAP-ApoE has longer expression, lower immune reactivity, and glial cell specificity.

3. MATERIALS AND METHODS

3.1. Animals and viral injection

Nine to ten month-old C57BL mice (purchased from the National Institutes of Health breeding facility in Indiana) and APP/PS1 double mutated AD mice (gift from D. Borchelt, Johns Hopkins University) were carried for these studies. AD mice were bred within the animal facility at the University of Chicago. All surgical and care procedures were pre-approved by the University Institutional Animal Care and Use Committee.

Animals were anesthetized intraperitoneally with ketamine (100 mg/kg) and xylazine (10 mg/kg), and then mounted in a mouse stereotaxic device (David Kopf, Inc). Under aseptic conditions, a small incision was made over the skull, and a burr hole was placed to allow entry of the injector. The injector consisted of a 30-gauge hypodermic needle connected to PE 10 tubing and a Hamilton 5 µl syringe. 2-3 ul of 10^9 infectious particles of recombinant Ad or AAV were stereotaxically injected into the hippocampus. Animals were allowed to survive for 1 week to 12 months, after which time they were overdosed with 100 mg/kg sodium pentobarbital i.p., then perfused transcardially with 0.9% saline followed by cold 4% paraformaldehyde. Brains were postfixed with 30% sucrose overnight, and then sectioned in 20-25µm sections using a sledge microtome.

3.2. Immunohistochemistry

Mouse brain sections were blocked in 10% normal serum/0.4% TritonX-100, followed by overnight exposure at 4^{o}C to the primary antibodies. Standard staining techniques for biotinylated and FITC- or RITC-conjungated secondary antibodies were used (40). The following primary antibodies were used: rabbit polyclonal antibody against GFP (1:2000; Clontech Laboratories, Palo Alto, CA), polyclonal anti-CD45 (1:500; Santa Cruz Biotechnology, Santa Cruz, CA), polyclonal anti-MHC class I (1:1000; Santa Cruz), rabbit polyclonal antibodies against ApoE2 and E3 (1:10000; gifts from G. Getz and K. Reardon, U. of Chicago), mouse monoclonal antibody against glial fibrillary acidic protein (GFAP, 1:1000; Sigma, St. Louis MO), and goat anti-human ApoE (1:5000, Chemicon). Double immunostaining was carried out with anti-GFP and anti-GFAP antibodies. Secondary antibodies included fluorescein isothocyanate (FITC)-conjugated goat-anti-rabbit IgG (1:50, Sigma), rhodamine-conjugated rabbit anti-goat IgG (1:5000, Chemicon), and tetramethylrhodamine-5-(and-6-)-isothiocyanate-conjugated (TRITC) goat-anti-mouse IgG (1:100; Cappel, Organon Teknika Corp., Durham, NC).

Tissue sections were stained using an avidin-biotinylated enzyme complex (ABC kit, Vector Laboratories). Ad-LacZ-injected mouse brain sections were rinsed with 150 mM sodium phosphate pH 7.4 (PBS), fixed with 2% formaldehyde and 0.2% glutaraldehyde, and then reacted with X-gal at 1 mg/ml for 4-12 h at 37°C.

3.3. Construction of rAAV-GFAP-ApoE alleles

The AAV vector expressing green fluorescent protein (GFP) was generously provided by A. Peel (U. of Florida) (Peel et al., 1997). Ad-ApoE2, Ad-ApoE3, and Ad-ApoE4 alleles were generously provided by G. Getz and K. Reardon (U. of Chicago).

AAV-GFAP-ApoE 2, 3, and 4 were constructed by excising a 1.1 kb fragment of ApoE alleles 2, 3, or 4 from Ad-ApoE, and fused with a 2.2 kb GFAP promoter (gift from M Brenner, NIH). The CMV-GFP-neoR fragment in plasmid pTR/UF5 (gift from Alyson Peel, U of Florida) was removed with restriction enzymes EcoR I and BamH I, allowing ITRs to remain in the AAV vector. The fusion fragment, GFAP-ApoE-poly A, was then cloned into BamHI site of the AAV backbone plasmid pTR/UF5. For verification of the GFAP-ApoE-polyA fragment, the 2.2 kb GFAP promoter and 1.1 Kb of ApoE allele coding region and poly-Adenylation signal were sequenced to confirm correct cloning and sequence. The amplification of AAV-GFAP-ApoEs was performed by stable transformation of STABL2 cells. rAAV-GFAP-ApoE alleles were then generated at the University of North Carolina Vector Core using a helper-free system (12).

3.4. Cell culture, Immunostaining, and Western blots

C6 and C343 glioma cells were maintained in DMEM supplemented with 10% FBS in a 10% CO_2 incubator. Cells were mock-infected or infected with rAAV-GFAP-ApoE for 48h for transgene expression. Immunostaining of infected cells was performed as described previously (38, 39). Briefly, infected cells were rinsed with PBS and fixed with 4% paraformaldehyde for 30 min, washed with PBS, then blocked with 5% normal serum/0.25% TritonX-100 in PBS, followed by overnight exposure at 4^{o}C to rabbit polyclonal antibodies against ApoE2 and 3. Biotinylated goat-anti-rabbit IgG was used as the secondary antibody for 1h at room temperature. The reactions were developed with an ABC kit (Vector). For protein expression by Western blot, infected cell lysates were extracted and run on 9% SDS-PAGE gels and transferred onto PVDF membranes (Immobilon-P, Millipore, Bedford, MA), as described previously (38). The membrane was blocked with 5% dry milk overnight at 4°C then incubated with rabbit polyclonal anti-ApoE antibody for 2h at room temperature. The blot was washed with TBST and incubated with HRP-conjugated anti-rabbit IgG (Amersham, Arlington Heights, IL) for 1h, then

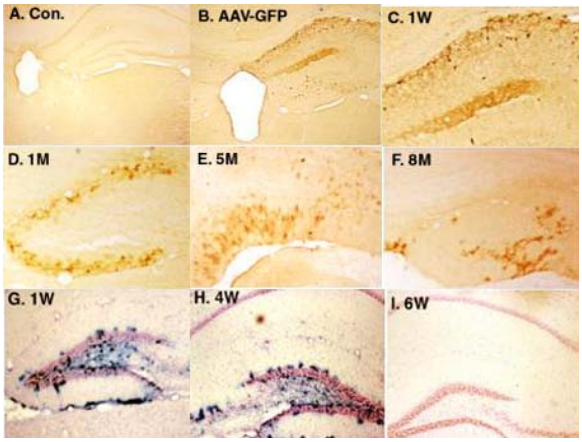

Figure 1. AAV vector induces long-term expression compared to Adenoviral vector in mouse hippocampus. 10^9 infectious particles of AAV-GFP and Adeno-LacZ were injected into the mouse hippocampus. Brain sections were stained with anti-GFP and X-gal for a time course study at 1 week, and 1, 5, or 8 months post-injection. Compared with PBS injection (A), stable expression in a representative AAV-GFP injected section (B) was detected with anti-GFP staining at 1 week, and 1, 5, 8 months (C-F). Ad-LacZ expression was detected with X-gal staining at 1, 4, and 6 weeks (G-I). Ad-LacZ expression declined with time and did not last longer than 6 weeks.

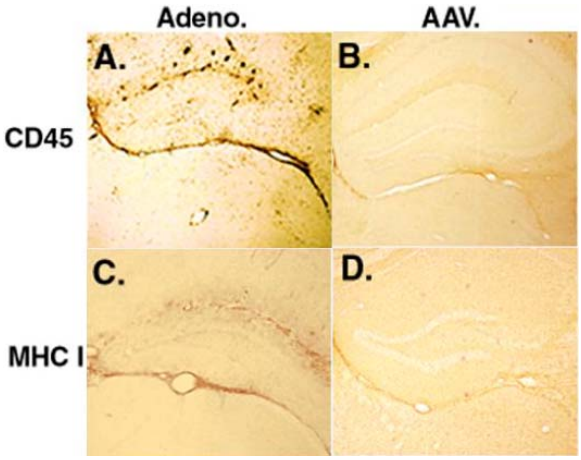

Figure 2. Adenoviral vector is more immunogenic than AAV vector. 10^9 infectious particles of Ad-ApoE and AAV-GFP were stereotaxically injected into the mouse hippocampus. The animals were perfused and fixed at 4 weeks post-injection and stained. Marked anti-CD45 immunoreactivity in Ad-ApoE3 injected mouse hippocampus (A) compared to AAV-GFP injected mouse hippocampus (B) was detected at 1 month post-injection. Anti-MHC class I staining was also stronger in Ad-ApoE injected mouse hippocampus (C) compared to AAV-GFP injected sections (D) at 1 month post-injection, indicating that adenoviral vector triggers higher immunoreactivity than the AAV vector.

visualized with enhanced chemilluminescence (ECL; Amersham).

4. RESULTS

4.1. rAAV vector mediates more stable expression than rAdenoviral vector in mouse hippocampus

Both Ad vectors and AAV vectors are highly effective vehicles for gene delivery in the CNS. We initially expressed Ad and AAV vectors with stereotaxic injection into normal aged mouse hippocampus to test the duration of transgene expression. Normal aged C57BL mice were stereotaxically injected with 10^9 infectious particles of AAV-GFP or Ad-LacZ into the hippocampus. Brain sections from mice sacrificed at 1 week, 1 month, 5 months, and 8 months post-injection were stained with anti-GFP.

Higher levels of AAV-GFP expression in AAV vector-injected mouse hippocampus were detected at 1 week post-injection (Figure 1B) as compared with PBS-injected controls (Figure 1A). Stable expression of AAV-GFP in hippocampus was also detected at 1, 5, and 8 months post-injection (Figure 1C-F).

In contrast, Adenovector-mediated LacZ expression were detected at high levels with X-gal staining at 1 week and 4 weeks (Figure 1G-H), but the expression of LacZ gene lasted less than 6 weeks (Figure 1I). Adenoviral vector mediated GFP or ApoE expression also significantly declined after 4 weeks, and could not be detected after 6 weeks (data not shown). These data demonstrate that AAV vector mediates longer and more stable expression than Adenoviral vector, which is possibly eliminated by the host immune response.

4.2. AAV vector is less immunogenic than adenoviral vector in mouse hippocampus

In order to compare the immune activity induced by Ad vector and AAV vector, aged C57BL mice were stereotaxically injected with 10^9 infectious viral particles of Ad-ApoE or AAV-GFP into the hippocampus. Brain sections from mouse brain at 4 week post-injection were then stained with antibodies to CD45, a leukocyte marker found on all cells of hematopoietic origin except erythrocytes, and to MHC class I, which is expressed in immune cells and microglia . Mouse brain sections of Ad-ApoE injected mouse exhibited expression of CD45 positive cells (Figure 2A) and MHC class I positive cells (Figure 2C). In animals injected with AAV-GFP, however, brain sections showed much less inflammation (Figure 2B or 2D). Ad-LacZ and Ad vector control injections in mouse hippocampus at 4 weeks also showed a marked immune response for CD45 and MHC I, similar to the response following the Ad-ApoE injection (data not shown). These results indicate that recombinant AAV is less immunogenic than that of Adenoviral vector, and the Adenoviral vector-induced immune response is independent of transgene expression. The AAV vector should therefore be a better choice for studying the biological or pathogenic function of transgenes for long-

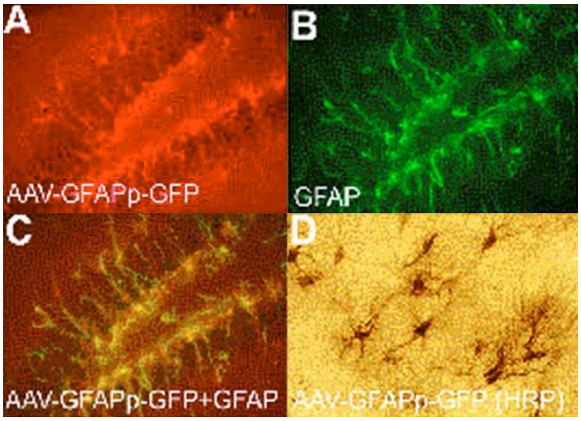

Figure 3. AAV-GFAP-GFP is specifically expressed in astrocytes of mouse hippocampus. Aged normal mice were stereotaxically injected with 10^9 infectious particles of AAV-GFAP-GFP into the hippocampus and assayed at 8.5 months. Glial cell specific GFP expression localized in astrocytes and stained with anti-GFP (A); expression of the GFAP promoter was detected with anti-GFAP antibody (B); GFP and GFAP co-localized based on anti-GFP and anti-GFAP double staining (C); GFP expression in astrocytes using HRP-conjugated secondary antibody with higher (40x) magnification is shown in 3D, indicating a typical astrocyte morphology.

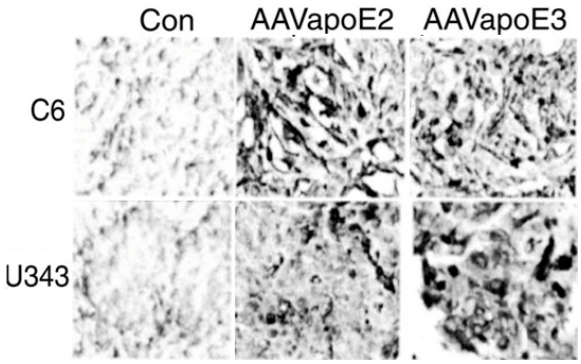

Figure 4. Expression of AAV-GFAP-ApoE alleles in glial cell lines. Glioma cell lines C6 and U343 were mock infected as a control (CON) or infected with AAV-GFAP-ApoE2 or ApoE3 for 48h. The cells were fixed and stained with anti-ApoE2 or anti-ApoE3. ApoE2 and ApoE3 were detected in infected cells but not in mock-infected controls, indicating that AAV-GFAP-GFP was expressed *in vitro*.

term and stable expression, such as investigating ApoE alleles in Alzheimer's disease..

4.3. Construction and expression of AAV-GFAP-ApoE alleles in astrocytes

In addition to evaluating immunogenicity and duration of expression, we studied cell-type specific expression for AAV vector transgenes, using human ApoE alleles which were highly expressed in astrocytes. To express ApoE alleles in a glia-specific manner in the transgenic mouse model of Alzheimer's disease, we first expressed an AAV-mediated GFP reporter gene in astrocytes by using the glial fibrillary acidic protein (GFAP) promoter, which consistently generates astrocyte-specific expression (11). Brain sections from mice at 8.5 months post-injection with AAV-GFAP-GFP were stained with anti-GFP (Figure 3A), anti-GFAP (Figure 3B), or double stained with anti-GFP and anti-GFAP antibodies (Figure 3C). GFP expression was restricted to astrocytes, clearly seen in higher magnification with typical astrocyte morphology (Figure 3D). GFP and GFAP colocalization, as shown in Figure 3C, indicated that AAV-GFAP-GFP had astrocyte-specific expression.

To generate AAV-GFAP-ApoE alleles, a series of cloning steps was employed. 1.1 kb ApoE alleles 2, 3, and 4 were fused with 2.2 kb GFAP promoter. The GFAP-ApoE-polyA cassette was then cloned into BamHI site of AAV backbone plasmid pTR/UF5. To characterize cloned GFAP-ApoE-polyA fragment, the 2.2 kb of GFAP promoter, 1.1 Kb of ApoE allele coding region and a poly-adenylation signal fragment were sequenced by automated sequencing. AAV-GFAP-ApoE constructs were stably transformed and amplified in STABL2 cells, a stable cell line for reducing recombinant mutations from pTR/UF5ITRs. Recombinant AAV-GFAP-ApoE viruses were subsequently generated at the University of North Carolina Vector Core using a helper-free system (12). The specific expression of ApoE alleles was verified in two glioma cell lines, C6 and U343. Glioma lines were infected with recombinant AAV-GFAP-ApoE viruses and protein expression was detected by immunohistochemistry with ApoE-specific antibodies (Figure 4).

4.4. Expression of AAV-GFAP-ApoE in hippocampus of Alzheimer's disease mice

To express these viruses in an Alzheimer's disease model, we used transgenic mice which over-express double mutations in the genes for APP and PS1 that are known to cause Alzheimer's disease in humans. Hippocampi of these transgenic mice were stereotaxically injected with 2 ul of 10^9 infectious particles of AAV-GFAP-ApoE2. After 12 months, ApoE was detected by immunofluorescent histochemistry with anti-human ApoE antibody. Compared to endogenous ApoE expression detectable in uninjected mouse brain sections (Figure 5A), a high level of AAV-GFAP-ApoE2 was detected in astrocytes after AAV-GFAP-ApoE injection (Figure 5B). This indicated that AAV-GFAP-ApoE was able to mediate long-term and stable expression in the transgenic mouse model for Alzheimer's disease.

5. DISCUSSION

ApoE alleles affect the pathogenesis of Alzheimer's disease, and may modify progression in MS and neurodegenerative diseases. In order to dissect the roles of ApoE isoforms in vivo, we used recombinant viral-mediated gene transfer to deliver different ApoE alleles into hippocampus cells. The results demonstrated that AAV vector mediated gene transfer has many advantages over Adenoviral vectors in regard to long-term expression, immunogenicity, and cell type specificity in normal aged

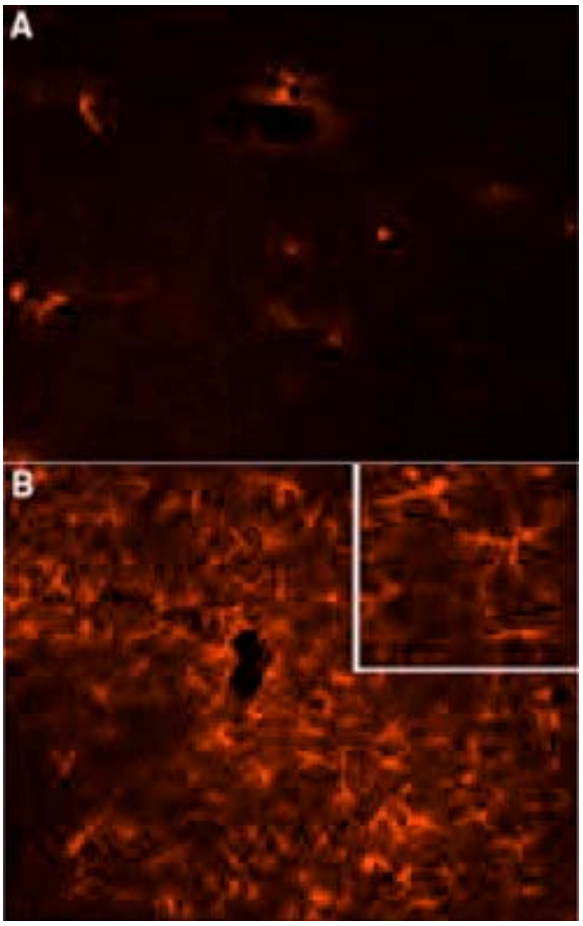

Figure 5. Long-term, astrocyte-specific expression of AAV-GFAP-ApoE in Alzheimer's disease mouse hippocampus. 10^9 infectious particles of AAV-GFAP-ApoE2 were injected into AD mouse (double mutations in APP and PS1) hippocampi for 12 months. After perfusion and fixation, mouse brain sections were stained with goat anti-human ApoE2, followed by rhodamine -conjugated anti-goat IgG. Endogenous ApoE staining was seen in PBS-injected control mouse brain (A). High level of ApoE2 is detected in astrocytes of AAV-GFAP-ApoE injected mouse hippocampus (B), indicating that AAV-GFAP-GFP expression is stable and astrocyte-specific.

mouse hippocampus as well as in the Alzheimer's disease mouse model.

Firstly, AAV vector-mediated reporter gene expression was long-term and stable in normal mouse hippocampus, compared to adenoviral vectors (Figure 1). AAV is a non-pathogenic virus that is capable of site-specific integration into chromosome 19 (13, 14, 15). All viral open reading frames in rAAV can be deleted, retaining only the inverted terminal repeat sequences for foreign gene expression (16, 17). The stability of expression may also be facilitated by chromosomal integration. (13, 19). The AAV vector can mediate highly efficient gene transfer into nondividing cells, including neurons and muscle cells, without inducing an inflammatory response *in vivo*. Thus, expression of foreign genes in the central nervous system is efficient and long-term, for up to one year (15, 21, 22, 23). As a result, AAV-mediated expression of foreign genes is stable, and no known pathogenic effects are reported to occur in animals or humans. Although Ad-mediated gene delivery has the advantage of providing high-level expression of transgenes in a wide range of hosts, transgene expression is transient. Adenoviral vectors have direct cytopathic effects, can elicit an immune response to viral and foreign transgene proteins, and can cause significant inflammation and rapid elimination of virally transduced cells.

Secondly, marked inflammation was induced by the Adenoviral vector, but not by the AAV vector, as seen in comparison of cellular immune responses in mouse hippocampus after viral vector directed gene delivery (Figure 2). The immune system plays a crucial role in limiting the duration of transgene expression from Ad vectors in peripheral organs as well as in the central nervous system (Kass-Eisler et al., 1994 and 1996). Systemically administered Ad vector is rapidly eliminated by innate immune defenses at the early phase (0-24 hour) of infection. Cytotoxic T lymphocytes also contribute to eliminating the Ad vector from liver of both normal and immunodeficient mouse (26, 27). Subsequently, transgenes decline within 21-30 days in mouse liver, muscle, and lung. Injection of Ad vector into mouse brain results in a slower decline of transgene (expression lasts up to 4-6 weeks). The Ad vector causes a significant inflammatory response. Even though the brain is an "immunologically privileged site," the Ad vector induces large numbers of T cells, macrophages, and activated microglia in and around the injection site, and MHC class I is upregulated (Figure 2C).

Cell-type specific AAV-mediated expression of ApoE, driven by the GFAP promoter, was characterized both in culture and in AD mouse hippocampi (Figures 3, 4, and 5). GFAP, an intermediate-filament protein expressed abundantly in astrocytes of the CNS, specifically promotes reporter gene and ApoE alleles in astrocytes with typical morphology. Transduced astrocytes near the injection site show greater numbers with GFAP than seen with other promoters, such as CMV (11, 28). Thus, the upregulation of GFAP gene activity that follows injury to the brain was mimicked by the transgene. Interestingly, AAV-GFAP-ApoE mediates high level of gene expression and lasts for at least 12 months in a transgenic mouse model for Alzheimer's disease (Figure 5). Viral strategies are excellent approaches to study the time course of various pathogenic mechanisms *in vivo*, to test cell-specific promoters, and to confirm the dose-dependency. Viral mediated gene transfer strategies are particularly appealing in studies of age-related cognitive decline or AD. Virus mediated gene transfers are likely to be effective approaches for gene therapy in the CNS as well as other organs *in vivo*.

In the CNS, ApoE isoforms are generated by glia, predominantly astrocytes (29, 30). ApoE affects neurite outgrowth (31, 32). In vivo studies with ApoE knock-out mice suggest that ApoE may play a role in structural plasticity during aging (33). ApoE also influences the

response to brain injury, possibly through alterations in neuronal repair mechanisms (34), and influences cell death after injury. In non-demented aged humans, ApoE immunoreactivity (IR) is present in blood vessels, astrocytes, and cortical neurons (36). Interestingly, patients with Alzheimer's disease have ApoE-IR in tangle-free as well as tangle-bearing neurons. Alzheimer's patients have lower levels of immunoreactive ApoE in the frontal cortex compared to aged non-demented controls, and there are lower levels of neuronal ApoE-IR in the brains of E4 familial Alzheimer's disease carriers (37) (E4 is associated with earlier age of onset and earlier cognitive decline). These discoveries raise the possibility that a disruption of ApoE pathways contributes to the pathology of Alzheimer's disease. GFAP-directed specific expression of ApoE in astrocytes by the AAV vector should provide a helpful tool for understanding the functions of ApoE, and help study the pathogenesis and gene therapy of Alzheimer's disease, and possibly other neurodegenerative diseases. Vectors with low immunogenicity are also likely to be required for therapy of neuroinflammatory disease such as multiple sclerosis.

6. ACKNOWLEDGMENTS

We acknowledge Dr. Alyson Peel from University of Florida for kindly providing pTR/UF5, an AAV vector. We appreciate Dr. Borchelt (Johns Hopkins University) who generously provided transgenic mice (APP/PS double mutation) for Alzheimer's disease and Dr. M Brenner from NIH for providing GFAP promoter. We also truly thank Dr. G. Getz from University of Chicago for providing ApoE plasmids.

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