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Adeno-associated virus serotypes 7 and 8 outperform serotype 9 in expressing atheroprotective human apoE3 from mouse skeletal muscle

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Abstract

Intramuscular injection of adeno-associated viral (AAV) vectors is potentially a safe, minimally invasive procedure for the long-term gene expression of circulating antiatherogenic proteins. Here, we compare secretion and atheroprotective effects of human apoE3 after injection of 3 pseudotyped AAV vectors (AAV2/7, AAV2/8, or AAV2/9), driven by the CMV enhancer/chicken β-actin (CAG) promoter, into skeletal muscle of hyperlipidemic apolipoprotein E–deficient (apoE$^{-/-}$) mice. Vector viabilities were verified by transducing cultured C2C12 mouse myotubes and assessing secretion of human apoE3 protein. Both hind limb tibialis anterior muscles of female C57BL/6 apoE$^{-/-}$ mice, 2 months old and fed a high-fat diet, were each injected with 1×10^{10} vector genomes of AAV vector. Identical noninjected mice served as controls; and blood was collected at weeks 0, 1, 2, 4, and 13. At termination (13 weeks), the brachiocephalic artery was excised; and after staining sections, plaque morphometry and fractional lipid content were quantified by computerized image analysis. Intramuscular injection of AAV2/7 and AAV2/8 vectors produced up to 2 μg human apoE3 per milliliter plasma, just below the threshold to reverse dyslipoproteinemia. AAV2/9 was notably less effective, mice having a 3-fold lower level of plasma apoE3 at 13 weeks and a 50% greater burden of atherosclerotic plaque lipid in their brachiocephalic arteries. We conclude that although vector refinement is needed to exploit fully apoE3 atheroprotective functions, AAV2/7 and AAV2/8 are promising gene transfer vectors for muscle-based expression of antiatherogenic circulating proteins.

1. Introduction

Human apolipoprotein E (apoE) is a 34-kd polymorphic glycoprotein, which plays a key role in plasma cholesterol homeostasis. The fully functional isoform, apoE3, has multiple atheroprotective properties, including receptor-mediated clearance of remnant lipoproteins and diverse lipid-independent antiatherogenic actions at the vessel wall [1]. Such protection is revealed indirectly in apoE-deficient (apoE$^{-/-}$) mice, which develop severe hypercholesterolemia and atherosclerosis [2].

Predictably, apoE3 is a prime candidate to alleviate atherosclerotic pathology. Initial reports of gene transfer used first-generation apoE3-expressing adenoviral vectors to target liver of apoE$^{-/-}$ mice; hyperlipidemia was transiently corrected before immune clearance of transduced hepatocytes [3]. Although improved adenoviral vector designs reduced toxicity and prolonged apoE expression [1,4,5], their potential to induce immune and inflammatory responses has prompted studies into alternative recombinant vectors. These include adeno-associated viruses (AAVs), which are nonpathogenic to humans and, compared with adenoviruses, elicit a relatively muted host response [6,7], a combination that offers potential for lasting transgene expression [8,9].

Previously, we reported that recombinant AAV-mediated apoE3 gene transfer to muscle inhibited progression of atherosclerosis in apoE$^{-/-}$ mice, despite producing only very low levels of plasma apoE3 (<10 ng/mL; 0.02% of normal) [10]. However, this study used archetypal AAV-serotype 2, which, although very efficient in vitro, is now recognized to

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have poor transduction efficiency in vivo [8,11,12]. Ultimately, productive apoE3 gene transfer by AAV vectors requires efficient transduction of target tissues by an optimal serotype. More than 100 variants of AAV have been identified in human or nonhuman primate tissues [8], of which 12 have been isolated and characterized for gene transfer [8,11-15]. Comparative studies have established that these exhibit tissue or cell-type tropism with often striking differences in transduction efficiencies [8,11-15].

Here, we assess three AAV vectors for muscle-based expression of atheroprotective human apoE3, all harboring the same AAV2-based expression cassette, but pseudotyped with capsids of AAV serotypes 7, 8, or 9. These serotypes were selected because they give high, persistent transduction of skeletal muscle by systemic or direct injection [12,13,16-19]; although early studies highlighted AAV1 effectiveness [11,12], this vector also transduces dendritic cells to invoke transgene-specific immune responses [18]. Notwithstanding, the immunologically distinct AAV2/7 and AAV2/8 vectors transduce skeletal muscle with efficiencies nearly equal to AAV2/1 [13], whereas AAV2/9 is a recent isolate from human tissue with high tropism for skeletal muscle [20] and, particularly, cardiac muscle [21,22]. Also relevant is the low level of neutralizing antibodies to AAV2/7 or AAV2/8 in human sera compared with AAV2/1 [13,23], while, intriguingly, it is possible that AAV2/9 vectors can be readministered even in the presence of neutralizing antibodies [24].

After vector validation in cultured cell lines, each of the 3 serotypes was injected into hind limb tibialis anterior muscles of young apoE$^{-/-}$ mice. Human apoE3 was readily detected in plasma at 1, 2, 4, and 13 weeks postinjection (0.4-2.1 μg/mL) and, although insufficient to reduce hypercholesterolemia, was highest in the AAV2/7- and AAV2/8-treated animals. Moreover, the mean plaque lipid content in their brachiocephalic arteries was two thirds the level in AAV2/9-injected mice, consistent with greater expression of atheroprotective apoE3.

2. Materials and methods

2.1. Production of AAV2/7, AAV2/8, and AAV2/9 vectors

Full-length human apoE3 complementary DNA [4] was ligated into pAAV2.CAG, a multiple cloning site *cis*-plasmid driven by the ubiquitous CMV enhancer/chicken β-actin (CAG) promoter [25]. Packaging of pAAV2.CAG. apoE3 into AAV particles was achieved by a triple plasmid transient transfection of HEK-293T cells, along with an adenovirus helper plasmid (pHGTI-adeno1) and a packaging plasmid containing the AAV *Rep* and *Cap* genes, at molar ratios of 1:3:1, respectively. To generate pseudotyped AAV vectors, the packaging constructs (pAAV7-2, pAAV8-2, or pAAV9-2) contained AAV2 *Rep* proteins, but *Cap* proteins from serotypes 7, 8, or 9. Cell lysates were prepared 2 days posttransfection, and AAV particles were purified by iodixanol discontinuous gradient ultracentrifugation [26]; viral titers were determined by DNA dot-blot hybridization analysis [27].

2.2. Transduction of cultured cells

HEK-293T cells (ATCC CRL-11268) were seeded overnight into a 6-well plate, whereas mouse C2C12 myoblasts (ATCC CRL-1722) were grown for 6 days to differentiate into mature myotubes. Culture medium was Dulbecco's modified Eagle's, supplemented with 10% fetal bovine serum and 2 mmol/L L-glutamine (Sigma, Dorset, United Kingdom). After removal of culture medium, serial dilutions of each AAV vector in serum-free medium (700 μL per well) were added; and the plates were incubated for 3 hours. Serum-containing culture medium was then substituted and analyzed at 24-hour intervals for secreted human apoE3 protein.

2.3. Intramuscular injection of AAV vectors

Animal experiments were carried out in 8-week-old female C57BL/6 apoE$^{-/-}$ mice (Charles River, Margate, United Kingdom). The studies were approved by the Ethics and Welfare Committee, Royal Holloway University of London, and conducted under statutory UK Home Office regulatory, ethical, and licensing procedures (the Animals [Scientific Procedures] Act 1986; PPL 70/6160). One week before injections, all animals were transferred to a high-fat (21% lard/0.15% cholesterol) diet until termination [28,29]. Mice were anesthetized with a 1:1 mix of fentanyl/fluanisone and midazolam, and then both hind limb tibialis anterior muscles were injected percutaneously with 1×10^{10} vector genomes (vg) of AAV vector. The control group was also composed of 8-week-old, fat-fed female mice, but was noninjected. Blood (up to 50 μL) was collected at weeks 0, 1, 2, and 4 by tail bleeds into heparinized capillary tubes and the plasma was separated by microcentrifugation at 8000*g* for 10 minutes at 4°C; at 13 weeks, blood was obtained by cardiac puncture under terminal anesthesia.

2.4. Analyses of culture medium and mouse plasma

Human apoE3 was detected in medium from transduced cells or in mouse plasma by Western blotting and quantified by a sandwich enzyme-linked immunosorbent assay (ELISA) using commercial reagents (Academy Biomedical Company, Huissen, the Netherlands) [4,10]. The capture antibody was polyclonal goat anti-human apoE (0.1 μg per well of a 96-well plate), whereas horseradish peroxidase–conjugated goat anti-human apoE antibody was used for detection (0.2 μg per well), adding tetramethylbenzidine before measurement. Dilutions of recombinant human apoE3 in apoE$^{-/-}$ mouse serum were used as standards. Total plasma cholesterol was measured in 96-well plates by mixing (1:100) with the Cholesterol Infinity reagent (Thermo Electron, Manchester, United Kingdom). Lipoprotein profiles in pooled plasma (10 μL) were analyzed by

agarose gel electrophoresis [4,10]; separated lipoprotein fractions were stained with Sudan black, and the relative amounts were estimated by scanning densitometry (Hyrys 2 Hit instrument with Phoresis software; Sebia UK, Camberley, United Kingdom).

2.5. Analyses of atherosclerotic plaque in brachiocephalic arteries

At termination, mice were perfused in situ with 10% formalin; and the brachiocephalic artery was excised with attached stumps of the aortic arch and right subclavian artery to aid orientation. Arteries were placed in liquid 2.5% agarose, proximal end downward, and, when solid, embedded in paraffin. Sections were cut every 30 μm, mounted, and stained with Miller elastin/van Gieson [28,29]. Analyses of plaque morphometry and fractional lipid content were performed with a computerized image analysis program (Image Pro-Plus; Media Cybernetics, Bestheda, MD) [29].

2.6. Statistics

Plasma human apoE3 and cholesterol concentrations for each group are shown as mean ± SD, whereas plaque measurements are given as mean ± SEM. Differences were analyzed by Student's t test; $P < .05$ was considered significant.

3. Results and discussion

3.1. Validation of vector viability

Each vector transduced HEK-293T cells efficiently, with the highest levels of human apoE3 protein occurring in the 24- and 48-hour medium collections and AAV2/7.CAG. apoE3 being the most potent vector (Fig. 1A). Transduction of mouse C2C12 myotubes with the AAV2/7 vector also produced markedly more secreted apoE3 than AAV2/8 or AAV2/9 vectors at 48 hours (Fig. 1B). Although these findings infer that gene transfer to muscle cells by AAV-serotype 7 is most efficient, there are currently no reports of similar serotype comparisons in vitro that could substantiate this conclusion. Vector validation in vivo was carried out by injecting both hind limb tibialis anterior muscles of 2 apoE$^{-/-}$ mice with 1×10^{10} vg of AAV2/7 and analyzing plasma periodically for human apoE3 protein. Western blotting and ELISA showed sustained expression with little reduction during the 27-week study (Fig. 1C).

3.2. Plasma levels of human apoE3 after intramuscular injection of AAV vectors

Three groups (n = 8) of 8-week-old, fat-fed female apoE$^{-/-}$ mice were injected intramuscularly with 1×10^{10} vg of each AAV vector, whereas a fourth (control) group was also fat fed but not injected. Plasma was collected before injection (week 0) and at 1, 2, 4, and 13 weeks postinjection and assayed for human apoE3 by immunoblotting and

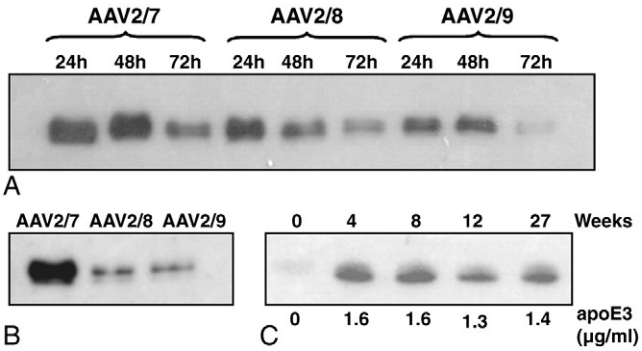

Fig. 1. Validation of AAV2/7, 2/8, and 2/9 vector viability. Recombinant viral preparations were added to HEK-293T cells and mature C2C12 myotubes in serum-free culture medium at a multiplicity of infection of 3.2×10^5 for 3 hours. Cells were then switched to serum-containing culture medium; and supernatants were collected 24, 48, and 72 hours later. ApoE3 protein (34 kd) secretion was evident by Western blotting in medium from HEK-293T cells (A; all time points) and mature C2C12 myotubes (B; only the 48-hour time point is shown). In vivo potency was demonstrated by injecting 1×10^{10} vg of AAV2/7 into both hind limb tibialis anterior muscles of apoE$^{-/-}$ mice and assaying plasma for human apoE3 protein at intervals up to 27 weeks. Western blotting confirmed secretion of human apoE3 into plasma, whereas ELISA (micrograms per milliliter) established that secretion was sustained throughout the study period (C).

ELISA. Plasma human apoE3 protein was detected in AAV2/7-, AAV2/8-, and AAV2/9-treated animals at each postinjection time by Western blotting, although the signal was noticeably weaker from the latter; no signal was produced from the plasma of control mice (data not shown).

Levels of plasma human apoE3 were measured in individual mice by ELISA and, for AAV2/7- and AAV2/8-treated animals, peaked at 1 week (1.9 ± 0.56 and 2.08 ± 0.43 μg/mL, respectively) (Fig. 2A, B, D). At 2 weeks, however, the concentration in AAV2/7-injected mice had declined markedly to 0.87 ± 0.47 μg/mL, which was significantly lower than the group receiving the AAV2/8 vector (1.78 ± 0.64 μg/mL, $P = .03$). This decrease, noted in each of the 8 mice treated with this vector, may reflect prompt sequestration of apoE3 by excess remnant lipoproteins and rapid hepatic clearance via interaction with low-density lipoprotein (LDL) receptor or LDL receptor–related protein. However, at 4 weeks, the level of human apoE3 had risen in the AAV2/7 group; and this was sustained until termination (13 weeks), when the concentration was comparable to that in the AAV2/8-treated mice (~1.4 μg/mL). This difference between vector serotypes may represent differential efficiency and kinetics of transduction and gene expression. One report suggests that AAV7 transduces skeletal muscle slightly better than AAV8 and equivalent to AAV1 [12], whereas, in a later study, pseudotyped AAV2/1, AAV2/7, and AAV2/8 vectors were all considered comparable [13]. However, the kinetics of AAV8 capsid dissociation and genome replication are reported to be faster than for other serotypes [30,31]. This may account for our observation that production of ApoE3 from AAV2/8 is higher in the first few weeks, thus being able to compensate for rapid apoE3

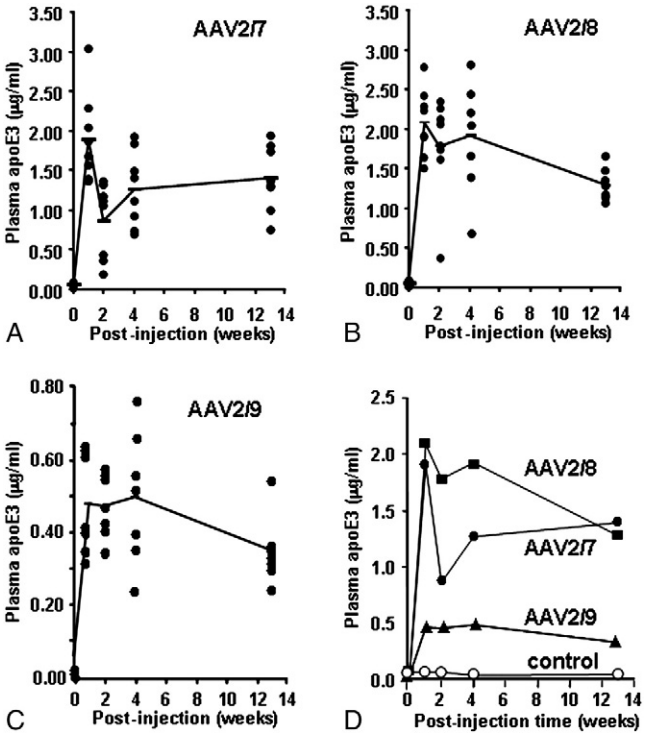

Fig. 2. Measurement of human apoE3 protein in plasma after intramuscular injections of AAV2/7, 2/8, and 2/9 vectors. Three groups of fat-fed female apoE$^{-/-}$ mice (n = 8) were injected with 1×10^{10} vg of AAV2/7.CAG.apoE3, AAV2/8.CAG.apoE3, or AAV2/9.CAG.apoE3 vector, whereas a fourth control group also fat fed was noninjected. Tail-vein bleeds were taken at weeks 0, 1, 2, 4, and 13; and plasma was assayed for human apoE3 protein. (A-C) Quantification by ELISA (micrograms per milliliter) of human apoE3 in mouse plasma after vector administration. Each point represents an individual mouse, whereas lines join the mean values for each time interval. Note that the y-axis scale is changed for the AAV2/9.CAG.apoE3 vector (C). For comparative purposes, mean values are also shown together for each vector and control mice (D); standard deviation bars are omitted for clarity.

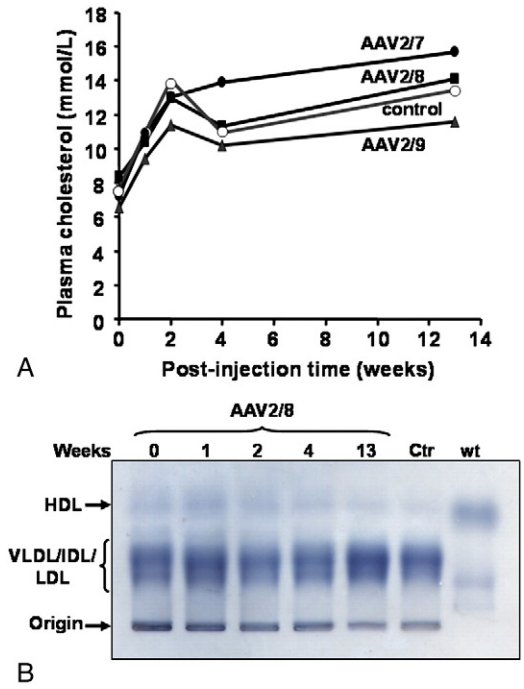

Fig. 3. Plasma total cholesterol and lipoprotein profiles after intramuscular injection of AAV2/7, 2/8, and 2/9 vectors expressing human apoE3. A, Total plasma cholesterol (millimoles per liter) was measured at each time point in individual fat-fed female apoE$^{-/-}$ mice receiving intramuscular injections of the vectors; control animals were also fat fed, but noninjected. Points represent mean values at each time, and standard deviation bars are omitted for clarity. B, Lipoprotein profiles of pooled plasma samples from AAV2/8-treated mice at each time point (0, 1, 2, 4, and 13 weeks). Lipoprotein fractions were separated by agarose gel electrophoresis and visualized by Sudan black staining of their lipid constituents. Ctr indicates control group at 13 weeks; wt, wild-type C57BL/6 mouse.

clearance, whereas the AAV2/7 vector increases ApoE3 production steadily over the 13-week period (Fig. 2D).

Levels of plasma human apoE3 in mice injected with AAV2/9.CAG.ApoE3 were lower than with AAV2/7 or AAV2/8, peaking at 0.49 ± 0.18 μg/mL, although this concentration was maintained throughout the study period (Fig. 2C, D). This was disappointing, as high efficiency was anticipated because AAV2/9 vectors robustly transduce skeletal muscle after systemic delivery [21]. However, our knowledge of AAV9 biology in vivo is still limited; and there are no previous reports of transgene expression by direct intramuscular injection of this serotype.

3.3. Hyperlipidemia is not ameliorated by intramuscular injection of AAV vectors

Total plasma cholesterol doubled in a linear manner between 0 and 2 weeks in both control and treated mice (Fig. 3A), a response most likely induced by the high-fat diet, although this was commenced 1 week before treatment [32-35]. At 4 weeks, however, cholesterol levels had declined by 20% in the control group, suggesting that alternative mechanisms for hepatic clearance of remnant lipoproteins may have been initiated in response to their excessive accumulation in plasma. Such mechanisms can include removal via scavenger receptor B1 [36,37] and apoE-independent uptake by LDL receptor and LDL receptor–related protein facilitated by lipoprotein lipase and hepatic lipase present in remnant surfaces [38]. Less-marked cholesterol decreases (between 8% and 15%) were also noted at 4 weeks in the AAV2/8- and AAV2/9-treated mice. From 4 to 13 weeks, plasma cholesterol showed modest increases for all groups; but there was no evidence in treated mice of circulating human apoE3 counteracting this rise, caused in part by aging as well as prolonged fat feeding (Fig. 3A).

The lipoprotein profile of pooled plasma from all groups was analyzed by agarose gel electrophoresis at each time point. The control apoE$^{-/-}$ mice, which received the same high-fat diet as the treated groups, showed high-density lipoprotein (HDL) as an α-migrating minor fraction, whereas very low-density lipoprotein (VLDL), intermediate-density lipoprotein, and LDL ran as a heavily stained broad pre-β

band; the origin was also heavily stained, as large VLDL and chylomicrons do not enter the gel (Fig. 3B). In contrast, plasma from normolipidemic wild-type C57BL/6 mice exhibit high HDL and a relatively low proportion of clearly separated VLDL and LDL. Scanning densitometry of lipoprotein profiles from AAV-treated apoE$^{-/-}$ mice did not reveal alleviation of the dyslipoproteinemia over time, including failure to increase the absolute amounts of HDL and the HDL to total lipoprotein ratio, which are surrogate indicators of effective human apoE3 gene transfer in apoE$^{-/-}$ mice [4] (Fig. 3B). The concentration of apoE reported to normalize plasma cholesterol in apoE$^{-/-}$ mice is approximately 2 μg/mL [39], which we achieved with AAV2/7 and AAV2/8 vectors at weeks 1, 2, and 4 in some individual mice (Fig. 2A, B). However, this threshold refers to mouse apoE, which has an enhanced ability to clear remnant lipoproteins compared with human apoE3 [40]. Presumably, this factor, exacerbated by the diet-induced rises in plasma cholesterol, explains why we were unable to detect diminution of remnant lipoproteins in our treated mice despite microgram amounts of human apoE3 protein being present in the circulation.

3.4. Atherosclerotic plaque development in brachiocephalic arteries

The apoE$^{-/-}$ mouse is a well-established model of hyperlipidemia and atherosclerosis, including the development of complex atherosclerotic lesions [2]. Moreover, in mice fed a high-fat, cholesterol-rich diet for over 2 months, the brachiocephalic (innominate) artery develops unstable plaques with a high frequency of rupture; and at later times, buried fibrous layers are evident that appear to mark healed ruptures [28,29]. Furthermore, plaques in mouse brachiocephalic arteries are more vulnerable when their lipid content is high [41], a feature of human plaque instability [42].

In agreement with these observations, our fat-fed apoE$^{-/-}$ mice also developed the same characteristic lesions, including lipid deposits and unstable plaques with visible buried fibrous caps (Fig. 4A). Unfortunately, plaque morphometric and lipid content data are presented only for treated mice (n = 7 per group) because of a technical problem during the processing of one batch of samples. However, as AAV2/9-injected mice had consistently low plasma human apoE3 (Fig. 2C), these animals were used as a reference group when analyzing data for effectiveness of AAV2/7 and AAV2/8 treatment, which produced 3-fold higher levels of plasma apoE3 (Fig. 2D). Thus, plaque area in AAV2/7- and AAV2/8-injected mice as a combined group was 23% lower than in the AAV2/9 group ($70.8 \pm 14.8 \times 10^3$ μm^2 vs $91.5 \pm 7.8 \times 10^3$ μm^2), although this decrease was not significant ($P = .16$), reflecting the large variability between individual mice in each group and the small number of animals used (Fig. 4B). Moreover, essentially plaque-free arteries were found in 3 of the mice treated with AAV2/7 or AAV2/8, whereas all arteries in the AAV2/9 group had lesions (examples in Fig. 4A). A more sensitive indicator of

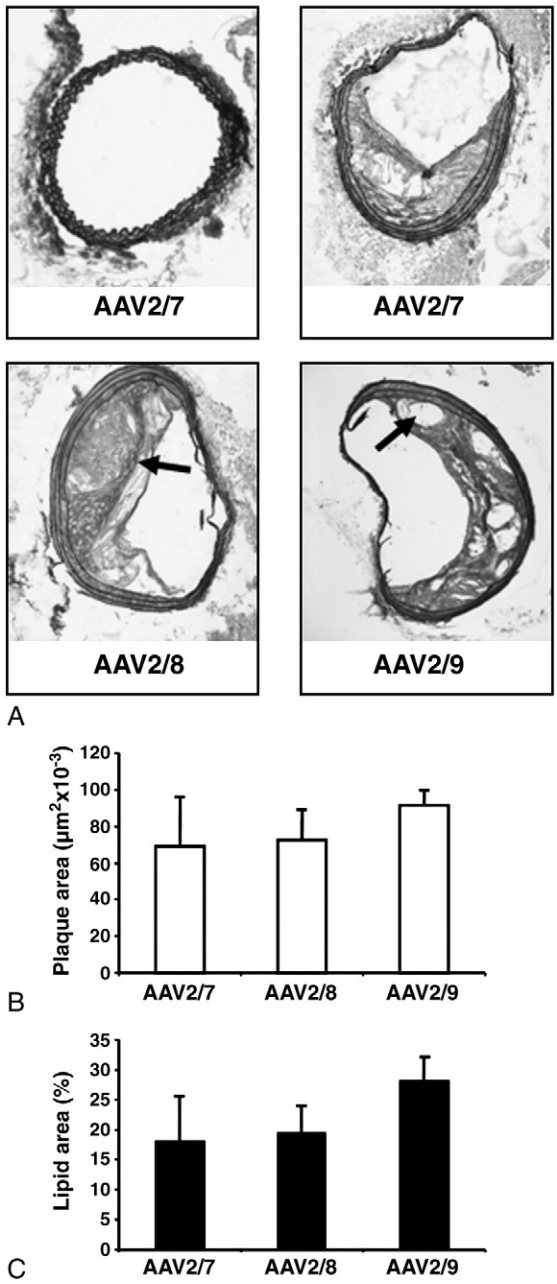

Fig. 4. Atherosclerotic plaque in the brachiocephalic arteries of fat-fed apoE$^{-/-}$ mice treated by intramuscular injection of human apoE3-expressing AAV vectors. Sections were stained with Miller elastin/van Gieson stain, which revealed variable plaque development. Three treated animals were essentially plaque-free (A; upper left, which shows an AAV2/7-injected mouse), whereas the other mice exhibited a range of lesions from modest to substantial. Plaque cross-sectional areas (B) and the lipid content within this area expressed as a percentage (C) were quantified using a computerized image analysis program (Image Pro-Plus). Data are presented as mean ± SEM, and a representative section from each group is shown in panel A: AAV2/7 (upper right, 21.5% lipid area); AAV2/8 (18.1% lipid area), and AAV2/9 (29.3% lipid area); an example of a buried fibrous cap is arrowed in the AAV2/8 section, whereas one of many lipid deposits is marked (arrow) in the AAV2/9 section.

treatment efficacy was plaque lipid content, as this was 50% higher in the AAV2/9 group (28.1% ± 4.1% vs 18.7% ± 4.2% in the AAV2/7 and AAV2/8 combined group, $P = .06$). Lipid content was not significantly different between the separate AAV2/7 and AAV2/8 groups (18.0% ± 7.5% vs 19.4% ± 4.5%, $P = .84$) (Fig. 4C).

In our previous study using AAV2/2 injections into apoE$^{-/-}$ mice fed normal chow [10], we quantified plaque by *en face* lipid staining of aortas and found significant inhibition of atherosclerotic lesion development, despite less than 10 ng/mL of human apoE3 in the circulation. Such aortic protection is consistently noted with alternative gene transfer vehicles that similarly deliver very low or undetectable plasma human apoE3 [1,39,43-45], apparently because apoE suppresses expression of adhesion and inflammatory molecules in early atherosclerotic lesions [46,47]. Disparate results are well documented when comparing atherosclerosis at different sites [48], and the brachiocephalic artery may possibly be less sensitive to antiatherogenic therapies. This might be compounded by differences in plaque development; lesions in the aortic sinus, for example, remain as fatty streaks for many months before fibrous caps can be discerned, whereas, in the brachiocephalic artery, they evolve rapidly to form advanced plaques in a few weeks, particularly if the apoE$^{-/-}$ mice are fat fed [29]. Conceivably, when only limited amounts of recombinant human apoE3 are secreted into plasma, their protective actions are overwhelmed attempting to control such rapidly growing lesions; circulating levels of human apoE3 higher than a few micrograms per milliliter are needed.

4. Conclusions

In summary, we have demonstrated that 2 pseudotyped AAV vectors (AAV2/7 and AAV2/8) efficiently transduce skeletal muscle to deliver, unlike archetypal AAV2, microgram quantities of antiatherogenic human apoE3 protein into the circulation. Moreover, this was achieved in female mice, which underexpress transgenes from liver-directed AAV vectors several-fold compared with males because of low androgen levels (at least for serotypes 2, 5, and 8) [49,50]. Although we report relatively modest atheroprotection, possibly because our analyses were confined to brachiocephalic arteries, this does not compromise our primary finding that transduction of skeletal muscle, a safe, accessible tissue, by productive AAV vectors delivers sustained secretion of an antiatherogenic protein. Nonetheless, vector refinement is needed to increase human apoE3 expression further so that its lipid-dependent and lipid-independent atheroprotective actions can be exploited fully [1].

Although not studied here, human apoE3 secretion may be reduced by host immune responses, directed against vector or transgene [8,10,17,51]. This can be minimized by using a muscle-specific promoter to prevent expression in antigen-presenting cells (and new regulatory cassettes promise high long-term transgene expression [52]) or by engineering AAV capsids to restrict tissue tropism [8,53]. Vector productivity can also be increased by codon optimization [54] and, for relatively short sequences, by the use of a self-complementary (scAAV) vector [54-56]. Such vectors package 2 complementary transgene cassettes into a single DNA molecule, which promotes rapid formation of transcriptionally active DNA duplexes after transduction of diverse tissues, including muscle [1,54-56]. Although the transgene cassette limit of 2.3 kilobases imposes severe constraints including use of truncated promoters [53], we recently demonstrated efficient hepatic expression of human apoE3 using an scAAV2/8 vector driven by a liver-specific promoter [57]. Clearly, such technological advances ensure future optimization of AAV vectors and offer realistic hope that intramuscular gene transfer of human apoE3 can treat atherosclerosis.

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References

[1] Harris JD, Evans V, Owen JS. ApoE gene therapy to treat hyperlipidemia and atherosclerosis. Curr Opin Mol Ther 2006;8:275-87.
[2] Meir KS, Leitersdorf E. Atherosclerosis in the apolipoprotein-E–deficient mouse: a decade of progress. Arterioscler Thromb Vasc Biol 2004;24:1006-14.
[3] Stevenson SC, Marshall-Neff J, Teng B, et al. Phenotypic correction of hypercholesterolemia in apoE-deficient mice by adenovirus-mediated in vivo gene transfer. Arterioscler Thromb Vasc Biol 1995;15:479-84.
[4] Harris JD, Graham IR, Schepelmann S, et al. Acute regression of advanced and retardation of early aortic atheroma in immunocompetent apolipoprotein-E (apoE) deficient mice by administration of a second generation [E1(−), E3(−), polymerase(−)] adenovirus vector expressing human apoE. Hum Mol Genet 2002;11:43-58.
[5] Kim IH, Jozkowicz A, Piedra PA. Lifetime correction of genetic deficiency in mice with a single injection of helper-dependent adenoviral vector. Proc Natl Acad Sci U S A 2005;98:13282-7.
[6] McCaffrey AP, Fawcett P, Nakai H, et al. The host response to adenovirus, helper-dependent adenovirus, and adeno-associated virus in mouse liver. Mol Ther 2008;16:931-41.
[7] Nayak S, Herzog RW. Progress and prospects: immune responses to viral vectors. Gene Ther 2010;17:295-304.
[8] Wu Z, Asokan A, Samulski RJ. Adeno-associated virus serotypes: vector toolkit for human gene therapy. Mol Ther 2006;14:316-27.
[9] Warrington Jr KH, Herzog RW. Treatment of human disease by adeno-associated viral gene transfer. Hum Genet 2006;119:571-603.
[10] Harris JD, Schepelmann S, Athanasopoulos T, et al. Inhibition of atherosclerosis in apolipoprotein-E–deficient mice following muscle transduction with adeno-associated virus vectors encoding human apolipoprotein-E. Gene Ther 2002;9:21-9.

[11] Chao H, Liu Y, Rabinowitz J, et al. Several log increase in therapeutic transgene delivery by distinct adeno-associated viral serotype vectors. Mol Ther 2000;2:619-23.
[12] Gao GP, Alvira MR, Wang L, et al. Novel adeno-associated viruses from rhesus monkeys as vectors for human gene therapy. Proc Natl Acad Sci U S A 2002;99:11854-9.
[13] Louboutin JP, Wang L, Wilson JM. Gene transfer into skeletal muscle using novel AAV serotypes. J Gene Med 2005;7:442-51.
[14] Mori S, Wang L, Takeuchi T, et al. Two novel adeno-associated viruses from cynomolgus monkey: pseudotyping characterization of capsid protein. Virology 2004;330:375-83.
[15] Schmidt M, Voutetakis A, Afione S, et al. Adeno-associated virus type 12 (AAV12): a novel AAV serotype with sialic acid- and heparan sulfate proteoglycan-independent transduction activity. J Virol 2008; 82:1399-406.
[16] Penaud-Budloo M, Le Guiner C, Nowrouzi A, et al. Adeno-associated virus vector genomes persist as episomal chromatin in primate muscle. J Virol 2008;82:7875-85.
[17] Sun B, Young SP, Li P, et al. Correction of multiple striated muscles in murine Pompe disease through adeno-associated virus-mediated gene therapy. Mol Ther 2008;16:1366-71.
[18] Lu Y, Song S. Distinct immune responses to transgene products from rAAV1 and rAAV8 vectors. Proc Natl Acad Sci U S A 2009;106: 17158-62.
[19] Zincarelli C, Soltys S, Rengo G, et al. Analysis of AAV serotypes 1-9 mediated gene expression and tropism in mice after systemic injection. Mol Ther 2008;16:1073-80.
[20] Gao G, Vandenberghe LH, Alvira MR, et al. Clades of adeno-associated viruses are widely disseminated in human tissues. J Virol 2004;78:6381-8.
[21] Inagaki K, Fuess S, Storm TA, et al. Robust systemic transduction with AAV9 vectors in mice: efficient global cardiac gene transfer superior to that of AAV8. Mol Ther 2006;14:45-53.
[22] Pacak CA, Mah CS, Thattaliyath BD, et al. Recombinant adeno-associated virus serotype 9 leads to preferential cardiac transduction in vivo. Circ Res 2006;99:e3-e9.
[23] Boutin S, Monteilhet V, Veron P, et al. Prevalence of serum IgG and neutralizing factors against adeno-associated virus types 1, 2, 5, 6, 8 and 9 in the healthy population: implications for gene therapy using AAV vectors. Hum Gene Ther 2010 [Epub ahead of print].
[24] Limberis MP, Wilson JM. Adeno-associated virus serotype 9 vectors transduce murine alveolar and nasal epithelia and can be readministered. Proc Natl Acad Sci U S A 2006;103:12993-8.
[25] Yue Y, Dongsheng D. Development of multiple cloning site *cis*-vectors for recombinant adeno-associated virus production. Biotechniques 2002;33:672-8.
[26] Zolotukhin S, Byrne BJ, Mason E, et al. Recombinant adeno-associated virus purification using novel methods improves infectious titer and yield. Gene Ther 1999;6:973-85.
[27] Snyder RO, Xiao X, Samulski RJ. Production of recombinant adeno-associated viral vectors. Current protocols in human genetics (John Wiley & Sons) 1996; 12.1.11-15.
[28] Johnson JL, Jackson CL. Atherosclerotic plaque rupture in the apolipoprotein E knockout mouse. Atherosclerosis 2001;154:399-406.
[29] Johnson J, Carson K, Williams H, et al. Plaque rupture after short periods of fat feeding in the apolipoprotein E–knockout mouse: model characterization and effects of pravastatin treatment. Circulation 2005; 111:1422-30.
[30] Thomas CE, Storm TA, Huang Z, et al. Rapid uncoating of vector genomes is the key to efficient liver transduction with pseudotyped adeno-associated virus vectors. J Virol 2004;78:3110-22.
[31] Akache B, Grimm D, Shen X, et al. A two-hybrid screen identifies cathepsins B and L as uncoating factors for adeno-associated virus 2 and 8. Mol Ther 2007;15:330-9.
[32] Plump AS, Smith JD, Hayek T, et al. Severe hypercholesterolemia and atherosclerosis in apolipoprotein E–deficient mice created by homologous recombination in ES cells. Cell 1992;71:343-53.
[33] Zhang SH, Reddick RL, Burkey B, et al. Diet-induced atherosclerosis in mice heterozygous and homozygous for apolipoprotein E gene disruption. J Clin Invest 1994;94:937-45.
[34] van Ree JH, Gijbels MJ, van den Broek WJ, et al. Atypical xanthomatosis in apolipoprotein E–deficient mice after cholesterol feeding. Atherosclerosis 1995;112:237-43.
[35] Sivak O, Darlington J, Gershkovich P, et al. Protonated nanostructured aluminosilicate (NSAS) reduces plasma cholesterol concentrations and atherosclerotic lesions in apolipoprotein E deficient mice fed a high cholesterol and high fat diet. Lipids Health Dis 2009;8:30.
[36] Fu T, Kozarsky KF, Borensztajn J. Overexpression of SR-BI by adenoviral vector reverses the fibrate induced hypercholesterolemia of apolipoprotein E–deficient mice. J Biol Chem 2003;278:52559-63.
[37] Van Eck M, Hoekstra M, Out R, et al. Scavenger receptor BI facilitates the metabolism of VLDL lipoproteins in vivo. J Lipid Res 2008;9: 136-46.
[38] Cooper AD. Hepatic uptake of chylomicron remnants. J Lipid Res 1997;38:2173-92.
[39] Thorngate FE, Rudel LL, Walzem RL, et al. Low levels of extrahepatic nonmacrophage ApoE inhibit atherosclerosis without correcting hypercholesterolemia in ApoE-deficient mice. Arterioscler Thromb Vasc Biol 2000;20:1939-45.
[40] Sullivan PM, Mezdour H, Aratani Y, et al. Targeted replacement of the mouse apolipoprotein E gene with the common human APOE3 allele enhances diet-induced hypercholesterolemia and atherosclerosis. J Biol Chem 1997;272:17972-80.
[41] Williams H, Johnson JL, Carson KG, et al. Characteristics of intact and ruptured atherosclerotic plaques in brachiocephalic arteries of apolipoprotein E knockout mice. Arterioscler Thromb Vasc Biol 2002;22:788-92.
[42] Virmani R, Kolodgie FD, Burke AP, et al. Lessons from sudden coronary death: a comprehensive morphological classification scheme for atherosclerotic lesions. Arterioscler Thromb Vasc Biol 2000;20: 1262-75.
[43] Cioffi L, Sturtz FG, Wittmer S, et al. A novel endothelial cell-based gene therapy platform for the in vivo delivery of apolipoprotein E. Gene Ther 1999;6:1153-9.
[44] Athanasopoulos T, Owen JS, Hassall D, et al. Intramuscular injection of a plasmid vector expressing human apolipoprotein E limits progression of xanthoma and aortic atheroma in apoE-deficient mice. Hum Mol Genet 2000;9:2545-51.
[45] Wientgen H, Thorngate FE, Omerhodzic S, et al. Subphysiologic apolipoprotein E (ApoE) plasma levels inhibit neointimal formation after arterial injury in ApoE-deficient mice. Arterioscler Thromb Vasc Biol 2004;24:1460-5.
[46] Stannard AK, Riddell DR, Sacre SM, et al. Cell-derived apolipoprotein E (ApoE) particles inhibit vascular cell adhesion molecule–1 (VCAM-1) expression in human endothelial cells. J Biol Chem 2001; 276:46011-6.
[47] Ma Y, Malbon CC, Williams DL, et al. Altered gene expression in early atherosclerosis is blocked by low level apolipoprotein E. PLoS One 2008;e2503:3.
[48] Bennett BJ, Wang SS, Wang X, et al. Genetic regulation of atherosclerotic plaque size and morphology in the innominate artery of hyperlipidemic mice. Arterioscler Thromb Vasc Biol 2009;29: 348-55.
[49] Davidoff AM, Ng CY, Zhou J, et al. Sex significantly influences transduction of murine liver by recombinant adeno-associated viral vectors through an androgen-dependent pathway. Blood 2003;122:480-8.
[50] Dane AP, Cunningham SC, Graf NS, et al. Sexually dimorphic patterns of episomal rAAV genome persistence in the adult mouse liver and correlation with hepatocellular proliferation. Mol Ther 2009;17:1548-54.
[51] Zaiss AK, Muruve DA. Immunity to adeno-associated virus vectors in animals and humans: a continued challenge. Gene Ther 2008;15:808-16.
[52] Salva MZ, Himeda CL, Tai PW, et al. Design of tissue-specific regulatory cassettes for high-level rAAV-mediated expression in skeletal and cardiac muscle. Mol Ther 2007;15:320-9.

[53] Li W, Asokan A, Wu Z, et al. Engineering and selection of shuffled AAV genomes: a new strategy for producing targeted biological nanoparticles. Mol Ther 2008;16:1252-60.
[54] Wu Z, Sun J, Zhang T, et al. Optimization of self-complementary AAV vectors for liver-directed expression results in sustained correction of hemophilia B at low vector dose. Mol Ther 2008;16: 280-9.
[55] McCarty DM, Fu H, Monahan PE, et al. Adeno-associated virus terminal repeat (TR) mutant generates self-complementary vectors to overcome the rate-limiting step to transduction in vivo. Gene Ther 2003;10:2112-8.
[56] Wang Z, Ma HI, Li J, Sun L, et al. Rapid and highly efficient transduction by double-stranded adeno-associated virus vectors in vitro and in vivo. Gene Ther 2003;10:2105-11.
[57] Osman E, Evans V, Graham IR, et al. Preliminary evaluation of a self-complementary AAV2/8 vector for hepatic gene transfer of human apoE3 to inhibit atherosclerotic lesion development in apoE-deficient mice. Atherosclerosis 2009;204:121-6.