Apolipoproteins A-I and A-V as gene therapeutic targets to intervene in lipid metabolism
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AAV gene therapy as a means to increase apolipoprotein A-I and high-density lipoprotein-cholesterol levels: Correction of murine apoA-I deficiency
Abstract

Inherited apolipoprotein A-I (apoA-I) deficiency is an orphan disorder characterized by high-density lipoprotein (HDL)-cholesterol deficiency and premature atherosclerosis. Constitutive overexpression of apoA-I might provide a means to treat this disease. This study provides a comprehensive evaluation of adeno-associated virus (AAV)-mediated apoA-I gene delivery to express human apoA-I and correct the low HDL-cholesterol phenotype associated with apoA-I deficiency. In an effort to maximize AAV-mediated gene expression, we performed head-to-head comparisons of rAAVs with pseudotyped capsids 1, 2, 6 and 8, administered by different routes with the use of five different liver-specific promoters in addition to CMV as single-stranded or as self-complementary (sc) AAV vectors. Intravenous administration of $1 \times 10^{13}$ gc/kg sc AAV8, in combination with the liver-specific promoter LP1, in female apoA-I$^{-/-}$ mice resulted in hApoA-I expression levels of $634 \pm 69$ mg/L, persisting for the duration of the study (15 weeks). This treatment resulted in full recovery of HDL-cholesterol levels with correction of HDL particle size and apolipoprotein composition. In addition, we observed increased adrenal cholesterol content and a significant increase in bodyweight in treated mice. This study demonstrates that systemic delivery of a sc AAV8 vector provides a means for efficient liver expression of human apoA-I, thereby correcting the lipid abnormalities associated with murine apoA-I deficiency. Importantly, this study demonstrates that AAV-based gene therapy can be used to express therapeutic proteins at a high level for a prolonged period of time and as such provides a basis for further development of this strategy to treat human apoA-I deficiency.

Introduction

Apolipoprotein A-I (apoA-I) constitutes the major structural protein of high-density lipoprotein (HDL). ApoA-I plays a crucial role in multiple steps of HDL metabolism: it is essential for the early lipidation of HDL by ATP binding cassette transporter A1 (ABCA1); it activates lecithin:cholesterol acyltransferase (LCAT) and is thereby crucial for further HDL maturation; it serves as a ligand for binding of HDL to scavenger receptor B1 (SRB1) for cellular cholesterol exchange processes. Moreover, apoA-I is reported to have intrinsic anti-oxidant and anti-inflammatory properties which have led to the development of apoA-I mimetic peptides,
investigational drugs that are currently tested for their potential to reduce atherosclerosis.\textsuperscript{1} The \textit{APOA1} gene is expressed in the liver and small intestine resulting in abundant apoA-I protein levels in the circulation. Inherited apoA-I deficiency is an orphan disorder that is characterized by HDL-cholesterol deficiency and premature atherosclerosis.\textsuperscript{2-4} To date, there is no effective treatment for this disease. Effective delivery and long-term expression of additional copies of human (h) apoA-I may provide a means to treat these patients. Several investigators have shown that transgenic overexpression of hApoA-I in atherosclerotic mouse models can reduce atherosclerosis progression.\textsuperscript{5-7} In addition, using a helper-dependent adenoviral vector, Oka and co-workers demonstrated that expression of hApoA-I resulted in long-term phenotypic correction of apoA-I deficient mice.\textsuperscript{8} To date, however, the most promising viral vectors for clinical application are recombinant adeno-associated viral (rAAV) vectors. These vectors are approved by the FDA for testing in phase I/II clinical trials, have not been associated with toxicity, and systemic administration does not lead to an acute innate immune response.\textsuperscript{9} To date, hundreds of patients have been treated with rAAV vectors without significant adverse effects.\textsuperscript{10} It is currently unknown what levels of AAV-delivered apoA-I are required to reverse the physiological effects of apoA-I deficiency in humans. Several factors contribute to AAV-mediated expression of transgenes. First, a number of AAV serotypes, differing only in their respective capsid proteins, have different tissue tropisms. Thus, the selected serotype directly affects transgene expression. Obviously, which tissue is targeted depends not solely on the employed AAV serotype, but also on the route of administration, the viral dosage, and the type of AAV genome that is used (single-stranded vs. self-complementary (sc) vectors). Besides these virus-related parameters, the employed promoter and/or post-transcriptional elements and/or the use of (natural) introns can greatly influence expression. Not only do promoters influence the level of expression, the use of tissue-specific promoters can also restrict expression to specific cells, which is a desirable feature in light of immune responses and safety in general. Taken together, to yield maximum expression for specific applications, in our case expressing apoA-I in the plasma compartment, a careful evaluation of the above-mentioned parameters was performed in this study. Prior to (pre)-clinical trials, we thus first evaluated whether optimizing the above-mentioned parameters could generate AAV vectors that are able to mediate sufficient hApoA-I expression to correct hypoalphalipoproteinemia in apoA-I deficient mice. Since it has been
shown that human apoA-I can be efficiently transferred to the murine liver\textsuperscript{5,6,11} and that this does not induce an immune response\textsuperscript{8}, we decided, in view of working towards clinical application, to use the human apoA-I variant for our studies. These results hold promise for further development and advocate the use of AAV vectors for efficacious long-term expression of therapeutic genes in the liver without significant side effects.

**Material and methods**

**Construction of vector plasmids**

Figure 1 shows the vectors that were used. First, to optimize AAV delivery, a construct was used that comprised the cytomegalovirus (CMV) promoter (access. nr. X03922, bp 621-1191) the coding sequence of human apoA-I (pre-pro-peptide, access. nr. NM_000039, bp. 39-839), the woodchuck post-transcriptional regulatory element (WPRE, access. nr. AY468486) and the bovine growth hormone poly(A) signal. Six additional vectors were produced to examine the effect of liver-specific promoters by exchanging the CMV promoter for the following promoter/enhancer combinations: 1) hLCAT-hApoA-I-WPRE containing the human LCAT promoter obtained by PCR amplification from human genomic DNA (access. nr. X04981, bp 1-836); 2) LSP-IVS-hApoA-I-WPRE containing the liver-specific-promoter LSP-IVS, consisting of two copies of the $\alpha_1$-micro-globulin/bikunin enhancer elements placed upstream of a minimal thyroid hormone-binding globulin promoter followed by a human $\beta$-globin intervening sequence (IVS) obtained from vector-plasmid p156RRLsinPPT-HPS-IVS-dFIX-PRE (a kind gift of Dr. I. Verma, Salk Institute, San Diego, CA, USA\textsuperscript{12}); 3) HCR-hAAT-hApoA-I-WPRE containing the human ApoE hepatic control region upstream of the human $\alpha_1$-antitrypsin promoter, which was isolated from ApoE*HCR-hAAT-FIX-pA (kind gift of Dr. M. Kay, Stanford University, Palo Alto, CA, USA\textsuperscript{13}); 4) AlbE-hAAT-hApoA-I-WPRE containing the human albumin enhancer element and the human $\alpha_1$-antitrypsin promoter (courtesy of and previously described by Kramer et al.\textsuperscript{14}) 5) rLFABP-IVS-hApoA-I-WPRE containing a truncated rat liver-fatty acid-binding protein promoter upstream of a chimeric intron isolated from pCI-FABP-p40 (courtesy Dr. J. Gordon, Washington University School of Medicine, St. Louis, MO, USA) 6) By excision of the WPRE from the latter construct, we also produced rLFABP-IVS-hApoA-I. In addition, two self-complementary (sc) AAV vectors were constructed to drive expression of human apoA-I. Sc
Figure 1. Schematic representation of the plasmid constructs used in the described studies. 1 CMV: Cytomegalovirus promoter; 2 hLCAT: human lecithin-acyltransferase promoter; 3 LSP-IVS: human thyroxine-binding globulin promoter, 2 alpha1-bikunin enhancers and an intervening sequence; 4 HCR-hAAT: ApoE-hepatic control region and the human alpha1-antitrypsin promoter; 5 Alb-enh-hAAT: albumin gene enhancer and the human alpha1-antitrypsin promoter, 6 rLFABP-IVS: rat liver fatty acid-binding protein promoter and intervening sequence; 7 LP1: minimized HCR-hAAT promoter/enhancer (see 4); 8 Synthetic enhancer and the human transthyretin promoter.

AAV vectors harbour a single-stranded DNA copy that consists of two copies of the transgene, with the second copy being an inverse repeat of the first, that are separated by a modified terminal repeat that allows refolding of the second copy onto the first copy, thus generating a double-stranded DNA molecule upon delivery into the host cell nucleus. The backbones of the two sc AAV vectors employed in these studies have been described previously.15,16 Briefly, the first sc AAV construct (courtesy of Dr. Nathwani, University College, London, UK) contains the LP1 promoter, a size-optimized version of the above-described ApoE*HCR-hAAT-promoter, followed by a SV40 intron16 whereas the second (courtesy of Dr. M. Kay) is driven by a synthetic enhancer and a minimized transthyretin (TTR) promoter.15 Both sc AAV vectors were constructed by replacing the
original coding sequences for FIX with the hApoA-I cDNA. Of note the WPRE element was not included due to size restrictions of the AAV vector.

**Virus production**

Recombinant AAVs were produced using a modified AAV2 genome and pseudotyped with capsids from AAV serotypes 1, 2, 6, and 8. The different AAV vectors are designated throughout this paper by the serotype of their capsids only. AAV1, 2, and 6 vectors were produced by transient transfection of 293 cells using the two-plasmid based system described by Grimm and co-workers. AAV8 vectors were produced by similar transient transfection using the 3-plasmid based system as described by Gao and co-workers. AAV vector particles were purified by iodixanol gradient centrifugation as described previously. Viral titres (in genome copies per ml) were determined using quantitative PCR (Q-PCR) with primers specific for the human apoA-I cDNA.

**Choice of AAV serotype, route of delivery and dose required for maximal CMV-driven liver expression of hapoA-I**

To evaluate AAV serotypes for their capacity to induce hepatic transgene expression (the main site for endogenous apoA-I production), AAV1, 2, 6 and 8 (transgenic cassette: CMV-hApoA-I-WPRE; Fig. 1) were injected via the hepatic portal veins of female C57Bl/6 mice. To this end, a ventral midline abdominal incision was made into the peritoneal cavity, and the portal vein was exposed. rAAV vectors (3x10^{12} gc/kg) were administered in a total volume of 200μL into the portal vein using a 31 gauge Ultra-Fine II Insulin syringe short needle. Hemostasis was achieved by application of a sterile cotton bud tip directly onto the site of injection. Blood samples were collected (after a 4 h fast) at 1 week prior to and 1, 3, 5 and 8 weeks post-vector administration. The mice were sacrificed at the 8-week time point.

Various administration routes were evaluated. Female C57Bl/6 mice received 3x10^{12} gc/kg bodyweight AAV8-CMV-hApoA-I-WPRE via the portal vein or tail vein (200μL), by intramuscular injections (in the gastrocnemius and adductor magnus muscles of both hind limbs; 50μL/injection site; n=4 sites total) or via oral gavage (200μL). The latter administration route was also evaluated for serotypes 1, 2 and 6 harbouring the same CMV-construct. We also evaluated dosage effects after intraportal injections of AAV8-CMV-hApoA-I-WPRE in C57Bl/6 animals using a dose range of 3 - 27x10^{12} gc/kg bodyweight.
Testing liver-specific promoter/enhancer combinations

Female LDLr-/- mice (anticipatory for future atherosclerosis studies) were injected via the tail vein with various liver-specific promoter/enhancer combinations (see Fig. 1) in single-stranded AAV8 vectors (dose: 3x10^{12} gc/kg bodyweight). In addition, we tested two sc AAV vectors as described above.

Phenotypic correction of apoA-I deficient mice

ApoA-I-/- mice were intravenously injected with sc AAV8-LP1-hApoA-I (see Fig. 1) at two doses (3x10^{12} gc/kg or 1x10^{13} gc/kg) whereas control animals were injected with phosphate-buffered saline (PBS). Fasted (4h) blood samples were collected 1, 3, 5, 8 and 12 weeks after virus administration and animals were sacrificed 15 weeks after virus administration.

Wild-type C57Bl/6 animals were obtained from Harlan (Horst, the Netherlands), whereas the LDLr-/- and apoA-I-/- animals (also on a C57Bl/6 background) were purchased from Jackson Laboratories (Bar Harbor, ME, USA) and subsequently bred at our facilities. Animals (12-20 weeks old) were housed in filter-top cages and had free access to water and regular chow (Hope farms, Woerden, the Netherlands). All animal experiments were approved by the local ethical committee on animal experimentation. Plasma from female wild-type C57Bl/6 mice was commercially obtained (Harlan) and was used as a wild-type reference for experiments with apoA-I-/- mice.

Plasma Analyses

The concentration of human apoA-I in murine plasma was determined by a sandwich ELISA using a rabbit-anti-human apoA-I antibody (Calbiochem, Gibbstown, NJ, USA) as a capture antibody and a monoclonal mouse-anti-human apoA-I antibody (Calbiochem) as a second (detection) antibody. The third antibody was a rabbit-anti-mouse IgG conjugated with horseradish peroxidase (Dako, Glostrup, Denmark) followed by colorimetry using TMB (Sigma, St. Louis, MO, USA). Human plasma with a known apoA-I concentration (measured with nephelometry) was used as standard. The assay had a minimum detection range of 2 ng/mL, an inter-
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assay variation of 8.3% and there was no cross-reactivity with murine apoA-I.
Fasted (4h) plasma samples were assayed for total cholesterol and HDL-chol-
sterol (after precipitation of apoB-containing particles using Lipidirect,
Polymedco, New York, NY, USA) using an established enzymatic method
(RTU, BioMérieux, Marcy L’Etoile, France). ApoB-depleted plasma samples
were also assayed for free cholesterol according to the manufacturer’s
instruction (Wako Chemicals, Richmond, VA, USA). Pooled plasma
samples (all animals of a group) were used to run fast protein liquid
cromatography (FPLC) profiles with online determination of cholesterol
as previously described.20 The same FPLC protocol was also used to collect
HDL fractions: 100μL of the pooled plasma was subjected to gel-filtration
and 630μL fractions were collected every 2 min from 41 to 55 min.
Subsequently, these fractions were used to detect human apoA-I, murine
apoE and murine apoA-II by western blotting employing specific
antibodies (Acris, Herford, Germany for human apoA-I and Santa Cruz,
Santa Cruz, CA, USA for murine apoE and A-II, respectively). Anti-AAV8
and anti-hApoA-I antibodies in pooled plasma samples were measured
essentially as previously described21 with the modification that plates were
coated with 100 μL AAV8 (1x10^{10} gc/mL) and human apoA-I (1 ug/mL,
Sigma), respectively. All measurements were performed on a single
microtitre plate and were expressed as LogEC50 of the O.D. after serial
dilution.

Tissue analyses

Liver tissue and the adrenal glands were homogenised in PBS using a
homogeniser (Fastprep 120, Bio101, Thermo-Savant, Holbrook, NY, USA).
Lipids were extracted as described previously.22 Total cholesterol in liver
homogenates was measured enzymatically (RTU, BioMérieux) whereas
total cholesterol in adrenal homogenates, because of the low levels of
cholesterol present, was measured using a highly sensitive fluorescent
enzymatic method, as described previously.23 Total cholesterol was
expressed per mg protein present in the tissue (BCA, Pierce, Rockford, IL,
USA). To quantify vector DNA copies in various organs after
administration of AAV vectors carrying the CMV-hApoA-I-WPRE
construct we performed quantitative real-time PCR with specific primers
for the CMV promoter, as previously described.21
Statistical analysis

All values are expressed as mean ± SD. Statistical significance, where appropriate, was determined using Student’s t-test and a $P < 0.05$ was considered statistically significant.

Results and discussion

Choice of AAV serotype, route of delivery and dose required for maximal CMV-driven liver expression of human apoA-I

Although initial AAV vectors based on serotype 2 had limited capacity to induce high protein expression levels, progress in vector development over the recent years has largely overcome this limitation. This progress prompted us to explore the potential of AAV gene therapy to correct apoA-I deficiency, one of the more abundant plasma proteins with normal plasma protein levels of 1000-1500 mg/L. The current analysis focused on achieving maximal AAV-induced plasma human apoA-I expression levels using all currently available tools at hand, i.e. using different AAV serotypes and routes of administration, optimizing viral dosage, use of different promoter/enhancer combinations, and evaluating the use of single-stranded versus self-complementary genomes. We only studied AAV gene transfer in vivo, since experience learned that in vitro evaluation of different serotypes and different AAV vector constructs does not reflect their efficacy in vivo.

The liver is the primary production site of apoA-I, thus we started our evaluation by evaluating CMV-driven plasma hApoA-I levels after intra-portal delivery of four different rAAV serotypes, i.e. 1, 2, 6 and 8. Recombinant AAV serotypes differ in their capsid proteins, resulting in different tissue tropisms. Administration into the portal vein of $3 \times 10^{12}$ gc/kg AAV encoding CMV-hApoA-I-WPRE (n = 4-6 animals per group) resulted in low plasma hApoA-I expression with rAAV1 and rAAV2, compared to AAV6 and especially AAV8, corroborating previous data\(^{18}\) (Fig. 2A). Being superior in this experiment, AAV8 was used in all subsequent experiments.

In search for the best administration route with this vector, animals were treated by oral gavage, intramuscular injection, intraportal injection, or tail vein injection with $3 \times 10^{12}$ gc/kg of rAAV8 (Fig. 2B). Oral gavage was tested to target the small intestine, a secondary site of endogenous apoA-I
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production. However, no hApoA-I was detected in plasma after oral administration of rAAV8 (Fig. 2B). This administration route was also evaluated for AAV1, 2 and 6; none of which resulted in detectable plasma hApoA-I levels either (data not shown). Moreover, Q-PCR on DNA isolated form different parts of the digestive tract (stomach, caecum, duodenum, ileum and colon) did not detect genomic copies of the CMV-hApoA-I-WPRE construct (data not shown).

Figure 2. Intravenous injection of AAV8 at a dose of 9x10^{12} gc/kg induces maximal human ApoA-I expression. Female C57/Bl6 mice were administered AAV-CMV-hApoA-I-WPRE to optimize AAV delivery. A) hApoA-I plasma levels of mice injected intraportally with 3x10^{12} gc/kg AAV of the indicated serotype. B) hApoA-I and C) genomic copy numbers of mice treated via the indicated route with AAV8 at a dosage of 3x10^{12} gc/kg. Q-PCR (Panel C) to assess DNA vector copy numbers was performed on DNA isolated from organs 8 weeks after injection. He; heart, lu.; lung, li.; liver, sp.; spleen, ki.; kidney, co.; colon, du.; duodenum, il.; ileum, ca.; caecum, st.; stomach. D) hApoA-I plasma levels of mice injected intraportally with rAAV8 at the indicated dosage. Data are from 4-6 (Panel A, B & D) or 3 (panel C) animals/group and are shown as mean ± SD.
It thus appears that these vectors were unable to transduce intestinal cells using oral gavage, corroborating previous data from Polyak and co-workers who also could not detect protein expression nor intestinal DNA copies after oral gavage of AAV vectors in mice. These findings contrast those of During and co-workers who showed that oral application of rAAV2 was able to correct lactose intolerance in rats, a discrepancy possibly explained by species differences. All other administration routes did result in detectable plasma hApoA-I expression. However, intraportal and tail vein injections induced higher expression compared to intramuscular delivery (at week 8; 12.4 ± 7.5 and 17.5 ± 16.0 mg/L for intraportal and tail vein delivery, respectively, compared to 2.2 ± 0.8 mg/L for intramuscular delivery; \( P < 0.05 \) for both systemic deliveries compared to intramuscular injection). Tail vein injection resulted in equal plasma levels of hApoA-I compared to intraportal vein injection. Additionally, evaluation of copy numbers of the CMV-hApoA-I-WPRE construct demonstrated a comparable distribution over the selected organs with an apparent preference for the liver after both systemic administration routes (Fig. 2C). These findings imply that complex surgical procedures to inject this specific viral vector into the portal vein are not necessary, at least in mice. Hence, we chose the less invasive tail vein injection as method of choice in subsequent experiments.

We subsequently evaluated dose-effects employing intravenous injection of rAAV8 with dosages of 3, 9 and \( 27 \times 10^{12} \) gc/kg, respectively, since limiting viral dose is desirable both for safety as well as for practical virus production reasons. Eight weeks after rAAV administration, plasma hApoA-I levels were significantly higher after treatment with \( 9 \times 10^{12} \) compared to \( 3 \times 10^{12} \) gc/kg (28.6 ± 2.5 vs. 11.5 ± 6.1 mg/L, \( P < 0.05 \)). The highest dose (\( 27 \times 10^{12} \) gc/kg), however, did not yield significantly higher expression compared to \( 9 \times 10^{12} \) gc/kg (35.5 ± 9.7 vs. 28.6 ± 2.5 mg/L, respectively, NS) hence the mid-dose was considered optimal (Fig. 2D). Taken together, intravenous delivery of rAAV serotype 8 at a dose of \( 9 \times 10^{12} \) gc/kg resulted in CMV-driven expression of hApoA-I amounting to plasma levels of approximately 30 mg/L.

**Liver-specific promoter/enhancer combinations increase human apoA-I expression when compared to the CMV promoter**

Although the immediate-early CMV promoter induces strong expression, tissue-specific promoters are considered more desirable, especially for
systemic delivery. In the case of apoA-I, we chose liver-specific promoters since the liver is an important natural site of expression. The advantage of using liver-specific promoters is that they primarily transduce hepatocytes thereby avoiding expression in antigen-presenting (Kupffer) cells. Additionally, liver-specific promoters might induce a desirable increase in expression compared to the CMV promoter, as has previously been suggested.\textsuperscript{14,26}

Figure 3. Optimizing transgenic cassettes increases human ApoA-I expression compared to the CMV promoter. Female LDLr\textsuperscript{-/-} mice were injected intravenously with 3×10\textsuperscript{12} gc/kg bodyweight AAV8. A) hApoI-I plasma levels of mice treated with the indicated single-stranded (ss) vector constructs. B) hApoA-I plasma levels of mice treated with the ss LFABP-IVS-hApoA-I construct with or without inclusion of the WPRE element C) hApoA-I plasma levels of mice treated with the indicated self-complementary (sc) AAV vectors. D) Anti-AAV8 antibody titers, data expressed as LogEC50 of the O.D. after serial dilution of pooled plasma samples of mice after injection of the indicated AAV constructs. Data (4-8 animals/group) are mean ± SD.
In this light we tested AAV8 vectors containing various transgenic cassettes with five liver-specific promoter/enhancer combinations (Fig. 1). The vectors were tested against the CMV-driven construct at a dose of 3x10^{12} gc/kg (Fig. 3A). Three to eight weeks after injection, all the tested liver-specific promoters induced much higher hApoA-I expression levels, ranging from 123.5 ± 7.7 mg/L with the LCAT construct to 231.9 ± 47.1 mg/L when using the HCR-hAAT-construct, compared to 30.6 ± 12.3 mg/L obtained with CMV-hApoA-I-WPRE (Fig. 3A).

We also evaluated the effect of the use of a WPRE element, since this element has been shown to enhance transgene expression in AAV vectors. Expression with the LFABP-IVS-hApoA-I-WPRE construct was > 2.5-fold higher compared to the identical construct without WPRE (192.2 ± 20.8 mg/L and 68.5 ± 13.5 mg/L, respectively, \( P < 0.05 \); Fig. 3B).

Taken together, intravenous administration of AAV serotype 8 at a dose of 3x10^{12} gc/kg bodyweight with a transgenic cassette containing the HCR-hAAT promoter/enhancer combination and inclusion of WPRE, yielded hApoA-I levels of 225 mg/L in LDLr−/− mice, signifying an ~8-fold improvement vs. expression induced with the CMV promoter.

**Self-complementary (sc) AAV vectors induce a higher expression compared to single-stranded (ss) AAV vectors**

The above described 8-fold increase of hApoA-I levels when using liver-specific promoter/enhancers compared to the CMV promoter was substantial, yet this was still below physiological levels in mice (225 vs. 600-700 mg/L). In recent years, several groups have genetically manipulated the single-stranded (ss) AAV DNA genome such that the expression cassettes can be packaged to form self-complementary (sc) dimers in individual AAV particles. These sc vectors have been shown not only to induce more rapid transgene expression after injection because they can fold into a transcriptionally active, double-stranded form immediately upon delivery into the target cell, but also to induce higher expression levels. The latter property is ascribed to the increased resistance to degradation of double-stranded DNA compared to ss DNA. Hence, we evaluated whether two different sc vectors, containing either the liver-specific promoter LP1 or the liver-specific transthyretin (TTR) promoter, could induce higher hApoA-I expression compared to the ss vectors tested so far. At a dose of 3x10^{12} gc/kg bodyweight both sc vectors produced 2-fold higher expression levels (5 weeks after AAV administration; 442.2 ± 49.2 and 414.0 ± 44.8 mg/L for sc LP1-hApoA-I and sc TTR-hApoA-I,
Optimization of hepatic AAV-mediated hApoA-I expression respectively), compared to our best-performing ss vector (HCR-hAAT-hApoA-I-WPRE; 207.6 ± 42.5 mg/L; Fig. 3A&C). Compared to previously published results\(^\text{16}\) this gain seems modest, but this might be related to the relatively high viral dose we used. In this respect, Nathwani and co-workers demonstrated that the better performance of sc vectors over ss vectors is more obvious when using relatively low viral doses.\(^\text{16}\) The absence of the WPRE element in both our sc constructs (due to size limitations) is likely a second factor that may have had an impact. As expected, pooled plasma samples of animals treated with AAV8-hApoA-I vectors tested positive for anti-AAV8 antibodies, with levels persisting for the duration of the study but without notable effect on transgene expression levels. There was no apparent difference in antibody titre between the ss vector and the sc vectors (Fig. 3D). Although apparently not influencing expression levels in the current experiment and not within the scope of this present study, it should be realized that this apparent immunogenicity of AAV capsid proteins, most likely, poses the biggest challenge for successful development of clinical applications for AAV. Especially since the vast majority of the human population has pre-existing antibodies against AAV capsid proteins caused by previous encounters.\(^\text{31}\) Clearly, this phenomenon warrants further research.

Taken together, AAV8 with a sc expression cassette driven by the liver-specific promoter LP1 resulted in a 200 to 250-fold amplification of hApoA-I transgene expression compared to the originally tested ss CMV-driven AAV2, reaching hApoA-I levels of 400-450 mg/L with a sub-optimal dose of 3x10\(^{12}\) gc/kg. Other investigators have previously reported expression of plasma hApoA-I levels up to 2000 mg/L\(^\text{5,6,8,32,33}\), yet these studies were all performed with adenoviral vectors that are considered less ideal for systemic clinical use because they evoke stronger host innate immune responses.\(^\text{34,35}\) In contrast, efforts to induce apoA-I expression with AAV provided disappointing results\(^\text{36,37}\), except for one.\(^\text{7}\) Lebherz and co-workers reported apoA-I expression levels of \(\approx650\) mg/L with a ss AAV8 vector at a dose of 1x10\(^{12}\) gc/animal, which translates to 4x10\(^{13}\) gc/kg.\(^\text{7}\) Although viral titers can not be reliably compared between laboratories, their described hApoA-I plasma expression levels in relation to viral titer appear to be in agreement with our results with ss vectors. However, as we show here, sc vectors can induce equal expression levels while using a lower viral titer and are therefore preferable since a lower viral dose is likely to result in a reduced immune response. Of note, throughout this evaluation we used female mice which are less amenable to AAV-mediated gene delivery, both
when using ss\textsuperscript{38,39} or sc vectors\textsuperscript{39,40}. Hence, the hApoA-I levels that could be achieved in mice using AAV might even be higher when using male instead of female mice.

**sc AAV8-LP1-hApoA-I induces phenotypic lipid correction in ApoA-I deficient mice**

The plasma hApoA-I levels that were achieved using either of the two sc AAV8 vectors were close to the levels seen in wild-type mice (600-700 mg/L\textsuperscript{6}). To study the physiological effects of sc AAV8-LP1-hApoA-I we used apoA-I deficient mice as a model for human apoA-I deficiency. These mice are characterised by decreased total and HDL-cholesterol levels, enlarged HDL particles and strongly altered HDL apolipoprotein composition, most notably an enrichment in apoE\textsuperscript{41-43}. ApoA-I deficiency in these animals appears to result in less functional HDL, i.e. a reduced cholesterol efflux capacity from atherosclerotic sites resulting in increased atherosclerosis and inflammation when crossed with LDLr\textsuperscript{-/-} mice\textsuperscript{44} and reduced cholesterol delivery to peripheral tissues\textsuperscript{41}. The latter influences in particular steriodogenic tissues which in mice are strongly dependent on HDL-mediated cholesterol delivery for steroid production\textsuperscript{45}.

ApoA-I\textsuperscript{-/-} animals (n = 5-6 per group) were injected IV with sc AAV8 containing the LP1-hApoA-I expression cassette at doses of 3x10\textsuperscript{12} or 1x10\textsuperscript{13} gc/kg bodyweight. Control animals were treated with an equal volume of PBS. Maximum hApoA-I plasma levels of 445 ± 43 and 634 ± 69 mg/L for the lower and higher dose, respectively, were reached at 8 weeks after vector administration with only slight decreases until 16 weeks (Fig. 4A). In contrast to the marked humoral immune response against the viral capsid proteins of AAV8 (Fig. 3D), we could not detect antibodies against hApoA-I in plasma in this experiment (data not shown) in accordance with Oka and co-workers who also could not detect an immune response to human apoA-I in apoA-I\textsuperscript{-/-} mice when using a helper-dependent adenoviral vector\textsuperscript{8}. In both situations, the introduced human variant of apoA-I is a neo-antigen. However, for mice to mount a humoral immune response against a protein requires expression in antigen-presenting cells\textsuperscript{46}. Using liver-specific promoters, it is probable that apoA-I is not expressed in these cells.

To assess the effects of hApoA-I expression on the cholesterol content of the main lipoprotein classes, we ran FPLC cholesterol profiles of pooled plasma samples 8 weeks after injection (Fig. 4B). For comparison we analysed a pooled plasma sample of untreated C57Bl/6 mice. On-line
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**Figure 4.** sc AAV8-LP1-hApoA-I induces phenotypic plasma lipid profile correction in ApoA-I deficient mice. ApoA-I/- mice were treated with PBS or with sc AAV8-LP1-hApoA-I at the indicated dose. As reference commercially obtained (Harlan) pooled plasma from wildtype C57Bl/6 animals was used. A) hApoA-I plasma levels after treatment with AAV8-LP1-hApoA-I (data are mean ± SD). B) FPLC profiles of online-measured cholesterol content of pooled plasma samples 8 weeks after treatment or reference plasma from wild-type animals. C) Western blots on HDL fractions derived from FPLC fractionation: Fr.1: min 41-43, Fr.2: min 43-45, Fr.3: min 45-47, Fr.4: min 47-49, Fr.5: min 49-51, Fr.6: min 51-53, Fr.7: min 53-55 and Fr.8: min 55-57. Data are from 5-6 mice per group.
cholesterol detection demonstrated that AAV-mediated hApoA-I expression led to a dose-dependent increase in HDL-cholesterol. In the highest dose group, we observed HDL-cholesterol levels that are normally seen in wild-type C57Bl/6 animals (Fig. 4B). This increase in HDL-cholesterol accounted for the observed increase in plasma total cholesterol levels. The FPLC profiles also showed an overall reduction of HDL particle size in animals expressing hApoA-I, as indicated by an increased column retention time of these particles. The peak retention time for the HDL fraction of PBS-treated apoA-I deficient animals was 47.0 minutes, whereas for animals treated with either dose (3x10^{12} or 1x10^{13} gc/kg) of sc AAV8-LP1-hApoA-I the retention time was 50.4, approaching the retention time seen for wild-type mice, i.e. 51.5 minutes. Furthermore, western blot analysis of collected HDL fractions (min 41-57 of the FPLC profiles) demonstrated that the human apoA-I was present in the collected HDL fractions (Fig. 4C). In line with observations of other investigators^41 we observed a marked apoE enrichment of the HDL fractions of apoA-I-/animals compared to those of wild-type mice, in which apoE was virtually undetectable by western blotting (Fig. 4C). Sc AAV8-hApoA-I administration markedly reduced the apoE content of HDL.

ApoA-II, the other major apolipoprotein on HDL, on the other hand, appeared to be only slightly decreased in HDL from untreated apoA-I-/animals compared to wild-type animals. Sc AAV8-hApoA-I injection appeared to increase apoA-II levels to comparable levels of untreated wild-type animals (Fig. 4C).

Quantification of cholesterol in total plasma and apoB-depleted plasma at 3 weeks after injection furthermore demonstrated that the increase of total plasma cholesterol induced by AAV-mediated expression of hApoA-I could be mainly attributed to an increase of esterified cholesterol in HDL (Table 1), suggesting that hApoA-I contributed to enhanced LCAT-mediated cholesterol esterification.

This latter suggestion is supported by data that hApoA-I activates murine LCAT even more effectively than murine apoA-I.^11 ApoA-I also serves as a ligand for binding of HDL to SRB1 for cholesterol delivery to peripheral tissues. Indeed, the AAV-induced expression of hApoA-I significantly increased the cholesterol content of the adrenals of AAV-hApoA-I-treated animals (Table 1). The increase of adrenal cholesterol content in our experiment was rather small compared to data reported by Plump and co-workers.^45 These investigators, however, compared wild-type animals with apoA-I-/animals, whereas we studied apoA-I-/mice that were treated with AAV-hApoA-I. The treatment of apoA-I-/deficient mice in
Table 1. Plasma and tissue lipid levels in ApoA-I deficient mice treated with PBS or sc AAV8-LP1-hApoA-I at the indicated dose

<table>
<thead>
<tr>
<th></th>
<th>Total plasma (Values in mg/dL)</th>
<th>HDL-fraction (mg/dL)</th>
<th>Liver (µg/mg)</th>
<th>Adr. (µg/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Human ApoA-I</td>
<td>TC</td>
<td>HDL-C</td>
<td>Non-HDL-C</td>
</tr>
<tr>
<td>PBS</td>
<td>-</td>
<td>47,8 (8,7)</td>
<td>29,7 (3,3)</td>
<td>18,0 (8,9)</td>
</tr>
<tr>
<td>Sc LP1-hApoA-I, 3x10^12 gc/kg</td>
<td>435,5 (30,5)</td>
<td>58,9 * (4,6)</td>
<td>41,7 * (4,6)</td>
<td>17,2 (5,8)</td>
</tr>
<tr>
<td>Sc LP1-hApoA-I, 1x10^13 gc/kg</td>
<td>621,5 (68,7)</td>
<td>72,8 * (13,1)</td>
<td>60,8 * (9,0)</td>
<td>12,0 (8,7)</td>
</tr>
</tbody>
</table>

Plasma lipid values were determined 3 weeks after treatment. TC indicates total cholesterol, CE indicates cholesteryl ester, FC indicates free cholesterol, Adr. indicates adrenals. Tissue cholesterol is expressed per mg protein. Data represent mean and SD (in parentheses) from 5-6 animals. *: Significantly different from PBS-treated animals.

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Adult life with AAV-hApoA-I may not be fully comparable to wild-type animals. The liver showed no increase in cholesterol upon the expression of hApoA-I (Table 1) but the impact of the AAV-induced changes in anabolism and catabolism of HDL on hepatic cholesterol content may only be evaluated by kinetic studies. Finally, we noted a significant increase in bodyweight in mice that were treated with the sc AAV-LP1-hApoA-I vector compared to controls (Fig. 5). To our knowledge, there exists no public literature that has evaluated natural bodyweight gain of apoA-I-/- mice and since the current study was not designed to unravel the underlying mechanisms we refrain from speculation on the metabolic background of this interesting observation at this point. Taken together, the collective data suggest that treating apoA-I-/- mice with AAV-hApoA-I improves the lipid profile with an apparent increase in HDL functionality.
**Figure 5.** AAV-induced hApoA-I expression in ApoA-I deficient mice increases bodyweight gain. ApoA-I-/- mice were treated IV either with sc AAV8-LP1-hApoA-I at the indicated dose or with PBS. Data are from 5-6 mice and are mean and SD. *: Significantly different from PBS-treated animals.

**Conclusions**

Our evaluation of AAV-mediated gene transfer (serotype, route of administration, dosage, and the use of ss vs. sc genomes) shows that optimizing these parameters for a specific application (in our case secretion of hApoA-I by the liver into the circulation) can yield high expression levels. Specifically, intravenous delivery of $1 \times 10^{13}$ gc/kg of sc AAV8-LP1-hApoA-I induced near-physiological plasma apoA-I levels of 500-600 mg/L for at least 15 weeks in female apoA-I-/- mice. Restoring apoA-I levels in this mouse model improved the plasma lipid profile with functional changes suggestive of improved HDL functionality. Combined with the ability of AAV8 to provide long-term transgene expression in the liver47, this approach holds promise for treatment of patients with congenital apoA-I deficiency who are at increased risk for atherosclerosis2-4 but for whom no treatment is available to date.

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References


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