Apolipoproteins A-I and A-V as gene therapeutic targets to intervene in lipid metabolism
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Lethal toxicity upon AAV-mediated green fluorescent protein expression in bone marrow transplanted mice precluded investigation of the cholesterol-efflux independent atheroprotective effects of apoA-I

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Abstract

Besides its atheroprotective role in reverse cholesterol transport (RCT), additional properties of apolipoprotein A-I (apoA-I), the principal protein of high-density lipoproteins (HDL), have recently received much attention. ApoA-I has, among others, anti-oxidative, anti-inflammatory and anti-coagulative properties. However, the contribution of these properties to the anti-atherogenic potential of apoA-I have thus far not been evaluated in vivo. In the current study, hepatic apoA-I production was induced in LDL receptor knockout (LDLr⁻/⁻) mice lacking adenosine-triphosphate binding cassette transporter A1 (ABCA1) in bone marrow-derived cells by treatment with adeno-associated virus (AAV) expressing human apoA-I under the control of a liver-specific promoter. This setup allowed us to study the atheroprotective effects of apoA-I upregulation independent of enhanced ABCA1-mediated cholesterol efflux from macrophages. Treatment of the wild-type and ABCA1 knockout transplanted animals with AAV to express hApoA-I was well-tolerated and resulted in supra-physiological levels of apoA-I. Surprisingly, treatment of the transplanted animals with a control vector expressing green fluorescent protein (GFP) was lethal within 3 weeks after injection. The unexpected and currently unexplained GFP toxicity under these conditions eliminated the control groups, thereby inhibiting a clear evaluation of the cholesterol-efflux-independent atheroprotective effects of apoA-I. Nevertheless, the employed strategy, i.e. induction of hepatic apoA-I expression in the context of selectively disrupted ABCA1-mediated cholesterol efflux, remains a promising tool to answer this question in the future.

Introduction

Plasma high-density lipoprotein (HDL)-cholesterol levels are inversely correlated with risk of coronary artery disease (CAD).¹⁻³ This association is mainly ascribed to HDL proteins, in particular apolipoprotein A-I (apoA-I). Indeed, among the parameters related to HDL, apoA-I seems to be the strongest independent risk factor for CAD.³ Traditionally, the atheroprotective function of HDL has been attributed to its role in the reverse cholesterol transport (RCT) pathway, i.e. the removal of excess peripheral cholesterol and its transport through lipoproteins back to the liver.⁴ Particularly relevant for atherosclerosis is the removal of excess cholesterol via RCT from macrophages in the arterial wall. Cellular cholesterol efflux,
the initial step of RCT, prevents these cells from developing into lipid-laden foam cells, a process generally regarded as the starting point of atherosclerotic lesion development. Critically important in this process is the action of adenosine-triphosphate binding cassette transporter A1 (ABCA1). Binding of lipid-poor apoA-I to ABCA1 stimulates cholesterol and phospholipid efflux from cells and results in the formation of discoidal HDL particles. Subsequently, the free cholesterol can be esterified to allow it to enter the core of the HDL particle, which, as a result, maturates into spherical HDL. HDL can subsequently deliver its cholesterol to the liver for excretion from the body.

Selective inhibition of cholesterol efflux from macrophages, by deletion of ABCA1, strongly increased diet-induced atherosclerotic lesion development in LDL receptor knock-out (LDLr/-) mice, demonstrating the critical role of this initial step in RCT for determining atherosclerosis susceptibility. The quantitative role of apoA-I in this process has proven difficult to establish in vivo because the absolute amount of cholesterol effluxed from peripheral macrophages is marginal compared to the amounts of cholesterol transported from the liver and the small intestine. However, using 3H-cholesterol labelled macrophage foam cells that were injected intraperitoneally into wild-type or apoA-I overexpressing mice, it was shown that apoA-I overexpression stimulated cholesterol efflux from these macrophages as witnessed by increased 3H-label excretion via the faeces of these animals compared to their wild-type counterparts. Taken together, there is strong evidence that ABCA1-mediated cholesterol efflux from peripheral macrophages protects against atherosclerotic lesion development in vivo and that apoA-I is essential in this process.

Over the last years other properties of apoA-I beyond its role in cholesterol efflux have gained considerable attention. ApoA-I has been reported to have anti-oxidative, anti-inflammatory and anti-coagulative properties that are thought to attenuate atherosclerosis. Several efforts have been made to demonstrate these properties of apoA-I in vivo using reconstituted HDL (rHDL), i.e. apoA-I complexed with phospholipids. Indeed, increasing circulating apoA-I levels by infusion of rHDL reduced markers of inflammation and oxidative stress in animal models for ischemia/reperfusion damage or collar-induced endothelial dysfunction. Given the early stages of endothelial damage, i.e. before actual lesion development in these models, it is unlikely that increased cholesterol efflux from recruited macrophages is involved in these protective effects. Thus, these data suggest that properties of apoA-I beyond its role in cholesterol efflux can decrease the initiation of
atherosclerotic lesion development. However, to what extent these apoA-I properties contribute to its over-all atheroprotective function in addition to its role in RCT is unclear. Increasing apoA-I levels, for example using gene therapy\textsuperscript{14-16}, has been shown to reduce atherosclerotic lesion formation in murine atherosclerosis models. In these experiments it is, however, impossible to discriminate between the beneficial effects of apoA-I on increasing cholesterol efflux from peripheral macrophages and additional protective effects. In an attempt to tackle this, the current study evaluates the effects of liver-directed apoA-I gene therapy in LDLr\textsuperscript{-/-} mice that were transplanted with ABCA1 deficient bone marrow.

**Methods**

**Virus production**

The human (h) apoA-I cDNA (access. nr. NM\_000039, bp. 39-839) including the prepro-sequence and the GFP cDNA were cloned into a self-complementary AAV vector with the strong and liver-specific promoter LP1, as described previously.\textsuperscript{17} AAV8 vectors were produced by transient transfection using the 3-plasmid based system as described by Gao and co-workers.\textsuperscript{18} AAV vector particles were purified by iodixanol gradient centrifugation as described previously.\textsuperscript{19} The viral titers (in genome copies per ml) of the viral batches were determined using quantitative PCR (Q-PCR) with primers specific for the LP1-promoter.

**Mice and Bone Marrow Transplantation**

Male LDLr\textsuperscript{-/-} mice were transplanted with bone marrow of ABCA1\textsuperscript{-/-} or wild-type (WT) littermates as previously described.\textsuperscript{8} Recipient LDLr\textsuperscript{-/-} mice (C57Bl/6 N5) were obtained from the Jackson Laboratory (Bar Harbor, ME, USA) and ABCA1\textsuperscript{-/-} donor animals (C57Bl/6 N8) were kindly provided by Dr. G. Chimini (Université de la Méditerranée, Marseille, France). All animals were bred at the Gorlaeus laboratories in Leiden, the Netherlands. To induce bone marrow aplasia, male LDLr\textsuperscript{-/-} mice were exposed to a single dose of 9 Gy (0.19 Gy/min, 200 kV, 4mA) total body irradiation using an Andrex smart 225 Röntgen source (YXLON International, Hamburg, Germany) with a 6-mm aluminium filter 1 day before transplantation. Bone marrow was isolated by flushing the femurs and tibias from male ABCA1\textsuperscript{-/-} or WT littermates. Irradiated LDLr\textsuperscript{-/-} recipients
received $0.5 \times 10^7$ ABCA1-/- or WT bone marrow cells by tail vein injection and will subsequently be termed ABCA1-M/M or WT animals, respectively. Mice were maintained on sterilized regular chow and were switched to a Western-type diet (WTD; 15% (w/w) fat and 0.25% (w/w) cholesterol (Diet W, Special diet Services, Whitham, UK) at 9 weeks after bone marrow transplantation (BMT) and kept on this diet for 8 more weeks. Drinking water was supplied with antibiotics (83 mg/L ciprofloxacin and 67 mg/L polymixin B sulphate) and sucrose (6.5 g/L). The experiment was approved by the ethics committee for animal experiments of Leiden University.

**AAV injection**

5 weeks after BMT, ABCA1-M/-M and WT animals received an intravenous (IV) injection of $7 \times 10^{12}$ gc/kg of AAV expressing either hApoA-I or GFP. 4 weeks after AAV administration a fasted (4h) blood sample was collected and animals were placed on the WTD for 9 weeks. Animals were sacrificed by exsanguination followed by whole-body perfusion after which the heart and other organs were collected.

**Plasma analyses**

Fasted (4h) plasma samples were assayed for total cholesterol and HDL-cholesterol (after precipitation of apoB-containing particles with PEG using an established enzymatic method (RTU, BioMérieux, Marcy L’Etoile, France). Pooled plasma samples (all animals of a group) were used to run size-exclusion chromatography profiles (FPLC on a Superose 6 column) with online determination of cholesterol as previously described. Plasma human apoA-I levels were measured on a COBAS-MIRA-analyzer (Roche Diagnostics, Basel, Switzerland). Plasma ALAT and ASAT were measured by standard techniques, based on Thelfeld's method, at the department of clinical chemistry of the Academic Medical Center in Amsterdam. Plasma IL-6 was measured using a sandwich ELISA (OptEIA, BD Biosciences, Franklin Lakes, NJ, USA) following the manufacturer’s protocol.

**Atherosclerotic lesion analysis**

Atherosclerotic lesion areas were assessed in Oil Red O-stained cryostat sections of the aortic root using a Leica image analysis system consisting of a Leica DMRE microscope coupled to a video camera and Leica Qwin
AAV-hApoA-I expression after selective knock-out of macrophage ABCA1 in LDLr⁻/⁻ mice

Imaging software (Leica Microsystems, Wetzlar, Germany). Mean lesion area (in μm²) was calculated from 10 consecutive sections, starting at the appearance of the tricuspid valves as described previously.⁸

**Statistical analyses**

Data are expressed as mean ± SEM. Where appropriate, statistical analyses were performed using the unpaired student’s *t* test and a *P* < 0.05 was considered significant.

**Figure 1. Experimental design.** One day before bone marrow transplantation (BMT) LDLr⁻/⁻ animals were exposed to total body irradiation and subsequently engrafted with 0.5 x 10⁷ bone marrow cells from ABCA1⁻/⁻ animals or from wild-type (WT) littermates (t=0). 5 Weeks after BMT, animals in both groups (WT and ABCA1⁻/⁻) were administered an intravenous injection of 7x10¹² gc/kg adeno-associated virus (AAV) to express either hApoA-I or GFP. 4 weeks after AAV administration i.e. 9 weeks after BMT, animals were switched to a Western-type diet (WTD) containing 15% fat and 0.25% cholesterol. After 9 weeks feeding WTD, animals were sacrificed for lesion assessment in the aortic root.
Results and Discussion

The experimental design of the study is depicted schematically in Fig. 1 and included four experimental groups of 11-13 male LDLr⁻/⁻ mice subjected to (1) BMT with ABCA1⁻/⁻ cells followed by AAV-hApoA-I, (2) BMT with ABCA1⁻/⁻ cells followed by AAV-GFP, (3) BMT with WT cells followed by AAV-hApoA-I, and (4) BMT with WT cells followed by AAV-GFP. In the groups treated with AAV-hApoA-I nearly all animals tolerated the AAV injections well. However, unexpectedly, the AAV-GFP-treated animals began to lose weight, became lethargic and started dying in the third week after AAV administration (Fig. 2), i.e. the moment when maximum transgene expression is reached.¹⁷

Figure 2. Survival curves of bone marrow transplanted animals after injection of AAV. Survival curves after respective AAV administration, i.e. 7x10¹² gc/kg of AAV-hApoA-I or AAV-GFP, in male LDLr⁻/⁻ mice having received bone marrow transplantation with wild-type (WT) or ABCA1⁻/⁻ cells (ABCA1⁻/⁻ mice).
These findings were unexpected since previous use of the same vector in apoA-I/-17 and LDLr/- mice (paper under review) did not reveal any detrimental effects. To examine the possible cause of the apparent fatality of AAV-GFP injection in BMT animals, it was decided to sacrifice the remaining AAV-GFP-treated animals (at that point four in the WT group and one in the ABCA1-M/-M group) and also, randomly chosen, three animals from both the AAV-hApoA-I-treated groups. Sacrifice of these animals was performed on day 23 post-AAV administration. The remaining AAV-hApoA-I-treated animals were studied according to the original experimental design as shown in Fig. 1.

Hepato-toxicity upon AAV-mediated GFP expression in bone marrow transplanted animals

AAV-GFP-treated animals showed a more extensive body weight loss at sacrifice on day 23 post-AAV administration (compared to one week before AAV injection) compared to their AAV-hApoA-I-treated counterparts (8.7 vs. 1.2 g., respectively, \( P = 0.003 \)). In addition, we observed slightly higher liver weight and reduced spleen weight, both absolute and relative to the bodyweight at the time of sacrifice, in AAV-GFP-treated animals compared to AAV-hApoA-I treated animals (Fig. 3B & C). ASAT and ALAT were found strongly elevated in all AAV-GFP-treated animals compared to the animals that had received AAV-hApoA-I (ALAT: 698.8 ± 426.6 and 40.3 ± 14.3 U/L, respectively, \( P = 0.025 \) and ASAT: 564.4 ± 259.7 and 110.7 ± 26.5 U/L, respectively, \( P = 0.017 \), Fig. 3D&E) but plasma IL-6 levels were in the normal range in all animals (Fig. 3F). In line, liver histology showed strong disorganization and destruction of hepatic tissue (Fig. 3G) in the AAV-GFP-treated animals, whereas the AAV-hApoA-I-treated animals showed normal liver morphology. Taken together, these data suggest that the hepatic expression of GFP was associated with cellular toxicity. Little is known about GFP toxicity although a few cases have been reported.21,22 On the other hand, GFP transgenic animals are viable and fertile without apparent gross problems23, suggesting that additional conditions must be met before GFP exerts toxic effects. Recently, Baens et al. described that GFP can inhibit polyubiquitination, a post-translational process which is responsible for tagging proteins to be degraded by the proteasome.24 Inhibition of this removal process upon GFP expression can lead to increased levels of detrimental proteins such as the p53 tumour suppressor that inhibits cell growth and promotes apoptosis, particularly under conditions of already increased levels of these proteins such as irradiation.
Figure 3. AAV-mediated GFP expression is hepatotoxic in bone marrow transplanted animals. Male LDLr−/− animals having received a bone marrow transplantation with WT or ABCA1−/− cells (ABCA1−/−M−/−M mice) were treated with AAV to express either hApoA-I or GFP. 23 Days after AAV injection, animals were sacrificed and bodyweight (A), liver (B) and spleen weight (C), plasma levels of liver enzymes ALAT (D) and ASAT (E), plasma IL-6 levels (F) and liver morphology (G) were analysed.

as used in the current experiment. However, analysis of p53 in the liver homogenates of AAV-GFP treated animals compared to AAV-hApoA-I-treated animals using Western blot did not demonstrate specifically increased levels of p53 in the livers of the AAV-GFP treated animals (data not shown). The observation that AAV-mediated overexpression of
hApoA-I in the liver of irradiated animals did not induce death suggests that the problems were caused by the expression of GFP in these animals. Additionally, although we can not exclude the possibility of quality differences between the AAV-GFP and AAV-hApoA-I batch, both viral batches were produced identically and no detrimental effects were observed with either batch when treating non-bone marrow transplanted LDLr\(^{-/-}\) mice (paper under review). On the other hand, these results may point at beneficial properties of apoA-I that protect the liver in this specific setup.

**Specific macrophage ABCA1 deletion under conditions of AAV-mediated hApoA-I overexpression results in a trend to enhanced lesion development**

Injection of AAV-ApoA-I resulted in both groups in comparable and (supra)-physiological levels of human apoA-I (4 weeks after AAV injection; 213 ± 9 and 223 ± 14 mg/dL in the WT and ABCA1\(^{-M/-M}\) group, respectively, compared to normal human physiological levels of 125 mg/dL, Fig. 4A). The hApoA-I levels reported here are higher than the maximum levels we reported previously using the same vector and dose.\(^{17}\) This discrepancy is likely related to the fact that in the current study we used male mice in stead of females. In agreement, others previously also reported better AAV-induced expression in males compared to females.\(^{25}\) Additionally, irradiation has also been reported to increase AAV-mediated expression\(^{26,27}\), although the 5 weeks time frame between irradiation and AAV injection makes this possibility less likely. Despite the supra-physiological levels of hApoA-I in these animals, we did not observe the anticipated increase in HDL-cholesterol, but instead a significant decrease of HDL-cholesterol levels in both AAV-hApoA-I treated groups at 4 weeks post-AAV administration compared to baseline (Fig 4B). However, other gene therapy studies also show only marginal increases in HDL-cholesterol with even higher human apoA-I levels\(^{14,16}\) while Lebherz et al. even showed a decrease in HDL-cholesterol when using AAV-mediated hApoA-I.\(^{28}\) In addition, to our knowledge, no literature is available on the use and effect of AAV in bone marrow transplanted animals. We therefore can not exclude that the viral AAV load in these by BMT immune-compromised animals may have caused a decrease in HDL-cholesterol, possibly masking an increasing effect by hApoA-I overexpression.
Figure 4. Macrophage ABCA1 deficiency, under conditions of hApoA-I overexpression, is atherogenic. Male LDLr⁻/⁻ animals received a bone marrow transplantation with WT or ABCA1⁻/⁻ cells and were subsequently treated with 7x10^{12} g.c./kg of AAV8-LP1-hApoA-I. Four weeks after AAV injection, animals were placed on a Western-type diet for 9 weeks and subsequently sacrificed, i.e. 13 weeks after AAV injection. A) Expressed plasma human apoA-I levels, B) Plasma HDL-cholesterol levels, C) Plasma total cholesterol levels, D) Cholesterol levels in lipoprotein fractions collected by FPLC fractionation of pooled plasma samples, E) Atherosclerotic lesion size. Data are expressed as mean ± SEM and statistical analyses were performed using the unpaired student's t test.

Four weeks after AAV injection, i.e. 9 weeks after BMT, the animals were placed on a Western-type diet (WTD). Both groups responded with an increase in total plasma cholesterol due to an increase in VLDL and LDL cholesterol levels (Figs. 4C and 4D). The increase, however, was less pronounced in the ABCA1⁻M⁻⁻ group, confirming previous results⁸ (Fig. 4C). In both groups, this increase in plasma total cholesterol was due to an increase in VLDL and LDL cholesterol levels (Fig. 4D). After nine weeks on the WTD, all animals were sacrificed to assess atherosclerosis development. As shown in Fig. 4E, a trend to larger atherosclerotic lesion areas was observed for mice reconstituted with ABCA1⁻/⁻ bone marrow compared to the animals transplanted with WT bone marrow (5.4 ± 3.1 x10⁵ vs. 2.4 ±
AAV-hApoA-I expression after selective knock-out of macrophage ABCA1 in LDLr−/− mice

1.2×10^5 μm^2, respectively, \( P = 0.07 \). This trend corroborates previous data showing that selective disruption of ABCA1 in bone marrow-derived cells significantly enhances the atherosclerosis susceptibility of LDLr−/− mice with normal murine apoA-I levels. The loss of both control groups (AAV-GFP-treated) unfortunately prevented a controlled study of the effect of hApoA-I overexpression in the presence or absence of macrophage ABCA1 on atherosclerosis development.

Correlation between human apoA-I concentrations and susceptibility to atherosclerotic lesion development

Using the data from the two remaining groups of animals that were treated with AAV-hApoA-I, we determined whether there was a correlation between serum hApoA-I concentrations and atherosclerotic lesion size (Fig. 5A). Interestingly, there was a strong negative correlation in the LDLr−/− mice that had received WT bone marrow (\( r = -0.64 \)). In the animals reconstituted with ABCA1−/− bone marrow, however, we did not find such a correlation (Fig. 5A). Based on these data one may conclude that increasing apoA-I is beneficial only in the situation where it can contribute to the process of cholesterol efflux at the level of macrophages in the vascular wall.

Further speculative analyses

Looking more closely at the individual mouse data, two animals in the ABCA1−M/−M group presented with unexpectedly small lesions, which were in the range of the WT group (Fig. 5A, arrows). Two actions in the protocol might have contributed to the large variation in the observed lesion sizes in the ABCA1−M/−M group; 1) the AAV-hApoA-I administration and 2) feeding the WTD. Fig. 5A (arrows) shows that hApoA-I expression levels of these two animals were in the range of the group, indicating that the surprisingly small lesions observed in these two animals were not the result of higher hApoA-I levels. Secondly, we analysed the response in plasma total cholesterol levels upon challenge with WTD. The two animals with the smallest lesions in the ABCA1−M/−M group presented with the lowest increase in plasma cholesterol levels when placed on the diet (Fig. 5B, arrows). The animals used for this BMT experiment were backcrossed 5 times to the C57Bl/6 background and thus it can not be completed excluded that the observed variation in response to diet between animals might be due to genetic differences. The primary objective of the study was to examine the effect of hApoA-I expression
Chapter 5

Figure 5. Correlations between plasma human apoA-I levels and atherosclerotic lesion size. Male LDLr−/− animals received a bone marrow transplantation with WT or ABCA1−/− cells and were subsequently treated with 7×10^{12} gc/kg of AAV8-LP1-hApoA-I. Four weeks after AAV injection, animals were placed on a Western-type diet (WTD) for 9 weeks and subsequently sacrificed, i.e. 13 weeks after AAV injection. A) Correlations between plasma hApoA-I levels and lesion size in all animals. Arrows indicate the 2 animals in the ABCA1−M/-M group with unexpectedly small lesions, B) Plasma cholesterol increase in the ABCA1−M/-M group after starting the WTD diet. Arrows indicate the 2 animals with the small lesions, C) Correlations between plasma hApoA-I levels and lesion size in all animals with exclusion of the 2 animals in the ABCA1-M/-M group with the unexpectedly small atherosclerotic lesions.

on atherosclerotic lesion development. In order to examine this question, the primary stimulus for lesion development in this model, i.e. plasma total cholesterol increase, should not vary too much within each group. To this end we decided to re-analyze the relationship between hApoA-I levels and atherosclerotic lesion development with exclusion of these two low responders to the WTD. We realize that this analysis is now prone to suffer
misinterpretations due to the very small numbers of animals in the remaining study groups (7 in the WT and 4 in the ABCA1\textsuperscript{M/-M} group) and should thus be interpreted with great caution. Despite this drawback, we nevertheless feel this approach might give some interesting thoughts on the role of apoA-I and hence deserves to be shown. Namely, once the two lowest responders to the WTD have been excluded, hApoA-I expression in both groups is negatively correlated to atherosclerotic lesion size (Fig. 5C). If this holds true, this would indicate that increasing apoA-I, the major protein constituent of HDL, can be beneficial even in a context of compromised ABCA1 function in macrophages. As such, this would demonstrate that properties of apoA-I beyond ABCA1-driven cholesterol efflux from macrophages contribute to athero-protection in vivo.

Conclusions and future directions

Due to the death of the AAV-GFP treated control groups and the low number of animals in the remaining groups (healthy animals were sacrificed at the time point of loss of the GFP groups), we were unable to draw firm conclusions on the cholesterol-efflux independent atheroprotective effects of apoA-I. However, we feel that the employed strategy, i.e. selective knock-out of ABCA1-mediated cholesterol efflux from peripheral macrophages in a context of apoA-I overexpression, is a promising strategy to answer this question in the future. For such experiments, however, it may be warranted to use apoA-I transgenic animals to increase plasma apoA-I levels in order to circumvent putative detrimental effects of treating bone marrow transplanted mice with AAV.

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References


AAV-hApoA-I expression after selective knock-out of macrophage ABCA1 in LDLr-/- mice


