Apolipoproteins A-I and A-V as gene therapeutic targets to intervene in lipid metabolism
Vaessen, S.F.C.

Citation for published version (APA):

General rights
It is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), other than for strictly personal, individual use, unless the work is under an open content license (like Creative Commons).

Disclaimer/Complaints regulations
If you believe that digital publication of certain material infringes any of your rights or (privacy) interests, please let the Library know, stating your reasons. In case of a legitimate complaint, the Library will make the material inaccessible and/or remove it from the website. Please Ask the Library: http://uba.uva.nl/en/contact, or a letter to: Library of the University of Amsterdam, Secretariat, Singel 425, 1012 WP Amsterdam, The Netherlands. You will be contacted as soon as possible.
Gene therapy in disorders of lipoprotein metabolism

S.F.C. Vaessen
J. Twisk
J.J.P. Kastelein
J.A. Kuivenhoven

Current Gene Therapy, 2007; 7: 35-47
Abstract

Current pharmacologic interventions in lipid metabolism are insufficient in a subset of patients at increased risk of cardiovascular disease. In particular, several monogenetic disorders of lipid metabolism with diverse clinical complications are beyond treatment to date. Somatic gene transfer is a potential approach to treat these disorders. This review describes the efforts made thus far to develop gene therapy for 3 major classes of dyslipidemia: Increased levels of low-density lipoprotein-cholesterol, reduced levels of high-density lipoprotein-cholesterol and increased plasma triglyceride levels. For many of the genetic causes underlying these conditions, proof-of-principle studies have been performed and in combination with improved vectors some of these strategies may be feasible for clinical use in the future.

Introduction

Disorders of lipoprotein metabolism are often implicated in proatherogenic processes which can ultimately result in cardiovascular disease. Increased levels of low-density lipoprotein cholesterol (LDL-cholesterol), reduced levels of high-density lipoprotein cholesterol (HDL-cholesterol) and increased plasma triglyceride (TG) levels are recognized as independent risk factors for cardiovascular morbidity and mortality. Pharmacologic interventions with statins (lowering plasma LDL-cholesterol levels), fibrates and bile sequestrants (lowering plasma TG) and niacin (increasing HDL-cholesterol levels) have been shown to confer significant favorable changes in terms of the cardiovascular risk profile. These drug classes are generally effective when the respective metabolic pathways are largely intact. In monogenetic disorders that result in complete deficiency of critical proteins in such pathways, however, these drugs are in broad terms ineffective. Homozygosity for loss of function mutations in key proteins of LDL, HDL or TG metabolism have been shown to underlie various dramatic phenotypes for which no treatment is available. In such cases, somatic gene transfer aimed at introducing the expression of wild-type proteins could theoretically offer a therapeutic solution. This review summarizes the efforts made in developing gene therapy to correct dyslipidemias thereby focusing on familial hypercholesterolemia (elevated LDL-cholesterol levels), hypoalphalipoproteinemia (low levels of HDL-cholesterol) and hypertriglyceridemia. For a more general review
on gene therapy for specific lipid disorders, we wish to refer to a recent review by Broedl and Rader.¹

Low-density lipoproteins

Genetic causes of elevated low-density lipoprotein-cholesterol

Two well-defined hereditary disorders cause elevations of LDL-cholesterol levels with consequent premature atherosclerosis and cardiovascular death. The best-known of these is familial hypercholesterolemia (FH; frequency 1:400 amongst Caucasians²) which is caused by LDL receptor deficiency. Especially homozygous FH causes massive elevation of LDL-cholesterol levels. Familial defective apolipoprotein B100 (FDB) constitutes the second but less frequent disorder, caused by loss of apoB100 function, a protein that is part of the LDL particle and serves as the ligand for the LDL receptor. Familial combined hyperlipidemia (FCH) constitutes a third disorder which, in addition to hypercholesterolemia, is also characterized by hypertriglyceridemia. However, the latter polygenetic disorder is not well-defined to date and lifestyle characteristics are known to play an important role in expression of the phenotype.

Statins are effective LDL-cholesterol lowering drugs that can reduce cardiovascular disease risk by 35%, however, these drugs can produce suboptimal effects in patients with homozygous FH.³ Current treatment for homozygous FH patients, therefore consists of repeated LDL apheresis or ectopic liver transplantation. For these cases, gene transfer strategies to induce LDL receptor expression or reduce apoB expression may bring relief especially since these individuals present with pronounced atherosclerosis and premature death. Gene therapies aimed at lowering LDL-cholesterol have focused on correcting homozygous FH, the obvious target group. If successful, these strategies might eventually also bring relief to other patients such as those suffering from heterozygous FH who don’t respond well to statins.

In the sections below we have discriminated between means directed at increasing LDL removal from the circulation and those that are aimed at reducing hepatic LDL production (see Fig. 1).
Review gene therapy and lipid disorders

A) Normal situation

B) LDL receptor deficiency (FH)

C) Inducing LDL receptor expression

D) Inducing VLDL receptor expression

E) Increasing ApoBect1

F) Decreasing ApoB mRNA

G) Increasing hepatic cholesterol removal
Figure 1. Schematic representation of proposed gene therapeutic strategies to correct LDL receptor deficiency, i.e. familial hypercholesterolemia. A) Normal situation and B) LDL receptor deficient situation. Proposed gene therapeutic strategies for correction: C) Overexpression of the LDL receptor increases uptake of LDL, D) Overexpression of the VLDL receptor increases uptake of LDL-precursor IDL, E) Reducing apoB100 secretion by inducing apoB editing. Consequently; less LDL is formed, instead apoB48-containing particles are formed which are probably removed by the liver via the LRP receptor, F) Inducing apoB mRNA degradation resulting in less LDL particles and G) Increasing hepatic cholesterol removal by overexpression of Cyp 7alpha-hydroxylase results in less LDL. White boxes, arrows and crosses represent gene therapeutic corrections and consequent effects.

Increasing LDL removal from the circulation

Overexpression of the LDL receptor

Non-viral strategies

Viral vectors have been shown effective in introducing foreign genes into host cells; however, with this strategy one also introduces viral capsid proteins that are able to evoke a host immune response in addition to the risk of viral replication and insertional mutagenesis. To circumvent this, non-viral vectors for DNA delivery to correct FH have been investigated since the beginning of gene therapeutic efforts. The general strategy is to complex DNA encoding the transgene to molecules with a high affinity for the target cells. Despite the above-mentioned advantages over viral vectors, non-viral strategies have their own particular drawbacks. Most important among these are the toxicity of the cationic complexing molecules, low transduction capacity and relatively short transgene expression due to lysosomal breakdown. For these reasons, only three relatively successful attempts to treat FH in this way have been described to date: In 1992, Wilson et al. described complexing the LDL receptor DNA with poly-L-lysine to asialoorosomucoid, a protein that has high affinity for the hepatic asialoglycoprotein receptor.4 Infusing such complexes into the ear vein of LDL receptor deficient rabbits, led to a 10 day liver-specific expression of the LDL receptor which in turn led to a 2 to 4% reconstitution of wild-type LDL receptor levels, yet causing a 20-30% decrease in plasma cholesterol levels. Other investigators used cationic liposome-DNA complexes attached to transferrin as the ligand.5 An intriguing feature of this approach was that gene transfer was directed at circulating leukocytes instead of hepatocytes thereby transforming the leukocyte pool into a sink for
cholesterol, which induced plasma cholesterol-lowering. However, correction was again incomplete and transient (21 days) probably due to the limited lifetime of circulating leukocytes. Razzini et al. used a hydrodynamics-based gene transfer approach to express a transferrin-LDL receptor fusion protein in the liver. With this method, a rapid infusion of a relatively large volume of plasmid DNA produces a hydrodynamic pressure that is associated with an increased uptake of plasmid DNA by hepatocytes. This approach resulted in expression of the fusion protein peaking at 18 to 24 hours post-injection and subsequent disappearance of expression after 120 hours. Using LDL receptor deficient mice, the secreted fusion protein was capable of binding LDL and mediating uptake of the complex by the liver. Consequently, the clearance rate of infused labelled LDL was normalized. Despite the drawback of low transduction efficacy and short-lived expression observed in this study, more advanced hydrodynamic approaches might, however, prove useful for some applications as recently reviewed by Miao et al.

To date, the problems of transient and low transgene expression using non-viral vectors have still not been overcome but non-viral vectors enjoy renewed attention due to the persisting challenges with immune responses when using viral vectors. Improvements to increase the potential use of non-viral vectors are therefore still ongoing and include among others reducing toxicity by structural modifications of the DNA-complexing molecules, improving transfection efficiency by increasing the vector circulation time, improving tissue-specific targeting using virosomes and increasing persistence of expression using site-specific integration methods such as transposons and integrases.

Cell-based gene therapy using retrovirus
Several investigators have attempted to correct FH by using cell-based ex vivo gene therapy, i.e. transplanting in vitro genotype-corrected cells into a deficient host. The first steps into this direction were already taken in 1986 when Kingsley et al. demonstrated that transfection of LDL receptor-deficient Chinese hamster ovary (CHO) cells in vitro with human DNA led to expression of the LDL receptor and corrected the multiple glycosylation defects of these cells. Subsequently, retroviral vectors introducing the LDL receptor gene were shown to transduce primary hepatocytes and fibroblasts isolated from the Watanabe heritable hyperlipidemic (WHHL) rabbit, a naturally-occurring animal model for FH. After introduction of the LDL receptor, these primary cells were able to take up and degrade LDL. In addition, this physiological correction resulted in decreased
intracellular cholesterol synthesis, an important mechanism in controlling intracellular cholesterol levels. In 1990, Wilson et al. took the next step and harvested hepatocytes from WHHL rabbits, cultured and infected them with recombinant retroviruses expressing the human LDL receptor gene and subsequently infused these cells into the portal veins of other WHHL recipients. All 7 recipients demonstrated significant decreases in serum cholesterol on days 2-6 following infusion, whereas animals treated with mock-infected hepatocytes showed no effect. However, from day 3 onwards, serum cholesterol levels slowly started to increase again reaching pre-treatment levels at day 6-8, most likely because of loss of the transplanted allogenic cells due to rejection. This thought was strengthened by data showing prolonged expression when using autologous fibroblasts or hepatocytes. Specifically, Chowdhury et al. used autologous hepatocytes and moreover transferred the rabbit LDL receptor instead of the human LDL receptor. This rendered stable presence of recombinant LDL receptor RNA in both liver and spleen for up to 6.5 months. Although only a 2 to 4% reconstitution of normal LDL receptor hepatic mRNA levels was achieved, this led to a striking 30% reduction of serum cholesterol levels. In combination with the lack of antibodies to the transfected rabbit LDL receptor, these data paved the road for a first gene therapy trial to correct FH in humans. Employing essentially the same technique, a total of 5 FH patients were treated with such LDL receptor gene therapy. Although the invasive procedures of partial hepatectomy and reinfusion of cells was tolerated quite well, serum cholesterol reductions were modest and metabolic responses after gene transfer varied considerably among the five recipients. As the authors stated: ‘Data from these 5 patients preclude a broader application of liver-directed gene therapy without modifications that consistently effect substantially greater gene transfer’. Although further efforts have been made to increase in vitro transduction efficiency, the invasive procedures combined with the development of new methodologies and viral vectors capable of efficient in vivo infection have excluded this method from clinical use to date.

One cell-based strategy, employing pluripotent stem cells of progenitor cells, still has perspective because these are non-immunogenic cells and thus can be allogenically transplanted. Only one such approach using human amniotic epithelial cells (HAECs) has currently been described as approach to increase LDL receptor expression. These investigators transduced HAECs in vitro with adenovirus expressing the LDL receptor and infused the manipulated cells into the ear vein of WHHL rabbits. Although a decrease in cholesterol levels was noted in all 3 treated animals,
which persisted for at least 20 days in one animal, most HAECs were shown to become part of the endothelial lining of the hepatic vessels and only a few engrafted within the host hepatic tissue. Nevertheless, the use of such non-immunogenic cells might offer possibilities for cell-based applications in the future and in particular human embryonic stem cells that still have the capacity to differentiate into various lineages.

**Systemic application of viral vectors: Adenoviral vectors**

The first studies into systemic gene delivery of the LDL receptor took advantage of the endogenous property of adenovirus to transduce hepatocytes with high efficiency in vivo. In mice, infusion of a first-generation adenovirus encoding the human LDL receptor induced within days high expression of the protein in predominantly hepatocytes and consequently normalization of the lipoprotein profile. However, these mice were sacrificed 4 days after virus injection, not permitting evaluation of persistence of gene expression. Using a similar approach but evaluating expression up to 20 days, an initial rapid drop in cholesterol levels was followed by a return to baseline levels within 2 weeks after administration of the virus. Overall, the systemic use of adenovirus-mediated gene transfer appeared to produce only transient effects due to innate as well as adaptive immune responses (both cellular and humoral) against viral as well as transgenic proteins. Furthermore, it was shown that a second infusion of adenovirus failed to induce transgene expression due to formation of neutralizing antibodies upon first contact with adenovirus. The latter poses another challenge since a high percentage of the general human population suffers from adenoviral infections and is therefore likely to have neutralizing antibodies. Taken together, early experiments with adenovirus demonstrate that in vivo gene transfer can lead to a substantial therapeutic effect but with several important challenges to overcome. Interestingly, these experiments also demonstrated additional beneficial effects of expressing the LDL receptor, such as a concomitant increase of HDL-C and apolipoprotein A-I. However, they also posed a warning: induction of supra-physiological levels of the LDL receptor led to massive, toxic cholesterol accumulation in hepatocytes supposedly due to the limited capacity of conversion of the sequestered cholesterol into bile acids.

To overcome some of the difficulties in these proof of principle studies with early viral vectors in the lipid field, Pakkanen and colleagues employed partial hepatectomy and ganciclovir to stimulate hepatic proliferation allowing in vivo infection by retroviral vectors, while Stein and co-
workers used strong anti-CD154 immunosuppression to enable prolonged expression with adenovirus. However, the development of better-equipped viral vectors, as discussed in the next paragraph, has dismissed further research and use of such drastic approaches.

Systemic application of viral vectors: helper-dependent adenoviral, lentiviral and adeno-associated viral vectors

Several viral vectors have been shown to be capable of inducing expression of the LDL receptor for several years and even life-long after one injection:

1) Helper-dependent adenovirus (Hd-Ad) also known as gutless adenovirus, are adenoviral vectors that lack the DNA sequences that encode for viral proteins. Eliminating all viral coding sequences prevents leak-through-expression of viral proteins that can evoke an adaptive immune response. Hd-Ad vectors thus offer decreased host immune responses to viral proteins, decreased cellular toxicity of viral proteins and increased capacity to accommodate larger expression cassettes (up to 36 kb of DNA) compared to first or second generation adenoviral vectors (in which the viral genes are only partially eliminated). However, producing these viral particles is challenging since it necessitates the use of helper adenovirus carrying all the coding regions for the viral particle structure. Hd-Ad viral vectors encoding for the LDL receptor have been shown to provide a life-long lipid reduction up to 90% and, importantly, reduced atherosclerotic burden in LDL receptor deficient mice. Untreated mice on an atherogenic diet for 20 weeks showed a 50% atherosclerotic lesion coverage of their aortas, whereas animals treated with Hd-Ad-LDLr were essentially lesion-free. When using the highest dose, however, 2 out of 8 animals developed antibodies against the transgenic product with a consequential loss of therapeutic effect. However, in the lower-dose group, which also showed a pronounced decrease in cholesterol levels, such antibody response did not develop.

2) Third-generation, self-inactivating lentiviral vectors with the use of liver-specific promoters. These vectors have also been reported to induce LDL receptor gene expression for at least two years in WHHL rabbits. These investigators reported a mean cholesterol decrease of 34% compared to animals treated with green fluorescent protein. Unfortunately, the effect of LDL receptor expression on atherosclerosis was not investigated. The vector was considered safe with regard to inflammation, hepatotoxicity and cancer. In contrast to Hd-Ad, lentiviral vectors do not require helper-virus and integrate stably into the genome of target cells, which can be both dividing and non-dividing cells. This integration property in combination
with liver-specific promoters allows for sustained expression but also increases the risk of insertional mutagenesis.

3) **Adeno-associated virus (AAV)** is a third vector that allows for long-term expression. Initial efforts using AAV serotype 2 (AAV2) with the LDL receptor gene driven by the CMV promoter produced low expression levels and induced an antibody response against the transgene in LDL receptor deficient mice that caused loss of transgene expression within 4 weeks.\(^{37}\) It has been suggested that the CMV promoter might either have acted as an adjuvant for antibody formation\(^{35}\) or allowed for expression in antigen-presenting cells.\(^{38}\) Indeed, use of AAV with the LDL receptor gene under control of a liver-specific promoter induced transgenic expression for at least 140 days without encountering expression-terminating immune responses.\(^{38}\) In addition, the use of novel AAV serotypes 7 and 8\(^ {39}\) was shown to increase hepatic expression of the LDL receptor compared to AAV2, resulting in a nearly complete normalization of lipid levels and an atherosclerosis reduction of approximately 50%.

Finally, Wade-Martins et al. recently proposed the use of a bacterial artificial chromosome introduced into high-capacity herpes-simplex virus (HSV).\(^ {40}\) They inserted the complete LDL receptor locus (LDL receptor gene with 45 kb of endogenous flanking sequences) into HSV, a virus which is capable of infecting a wide range of cells, at least in vitro. The inclusion of the complete gene locus allowed for physiological regulation of gene expression in vitro, e.g. sensitivity to intracellular cholesterol levels due to presence of sterol-responsive elements. Application of such a vector, if proven safe and efficacious, may be preferred above approaches using constitutive promoters.

**Overexpression of the VLDL Receptor**

An alternative to enhancing the removal of LDL particles is to stimulate the removal of their precursor particles in the circulation, i.e. intermediate-density lipoprotein (IDL) and very-low density lipoprotein (VLDL). In an effort to gain more insight in the biology of the then recently discovered VLDL receptor, Kobayashi et al. used adenovirus to overexpress the VLDL receptor in the liver of LDL receptor deficient mice.\(^ {41}\) In this model, they identified IDL as the preferred substrate for the VLDL receptor with apoE as the functional ligand for liver uptake. Overexpression of the VLDL receptor reduced levels of IDL and consequently LDL. These data supported the idea of exchanging the LDL receptor for the VLDL receptor in a gene therapy effort to treat FH (see Fig. 1D). The rationale for this is
quite strong since the VLDL receptor expression lasted longer compared to the human LDL receptor due to the fact that the VLDL receptor protein did not act as a neo-antigen in LDL receptor deficient mice. Using AAV2 or Hd-Ad for VLDL receptor gene delivery in LDL receptor deficient mice on a Western-type diet proved effective in decreasing IDL/LDL cholesterol levels and atherosclerosis. Importantly, increased expression of the VLDL receptor in combination with a low-fat diet regimen was even shown to induce regression of advanced atherosclerotic lesions. Of note, regression of atherosclerosis is of crucial importance for putative future treatment of FH patients with established disease.

**Decreasing LDL production**

**Decreasing levels of apoB100**

Another way of reducing LDL-C levels is to reduce LDL synthesis and/or secretion by the liver. Initial attempts to reduce apoB100 production made use of a natural-occurring mechanism that controls the fate of apoB100 mRNA. The *APOB* gene encodes both apolipoproteins B100 and B48. In the liver, apoB100 is produced from the complete transcript, whereas in intestinal cells the apoB100 mRNA is edited by an apoB mRNA-editing protein. This enzyme converts apoB100 mRNA into apoB48 mRNA by changing the first base of the glutamine codon (CAA) at residue 2153 causing a translational stop codon (UAA). In humans, the respective editing occurs exclusively in the intestine whereas rodents also have substantial expression of apoB mRNA-editing protein in the liver. Theoretically, up-regulation of apoB100 mRNA editing in the liver would thus lead to decreased synthesis of LDL (see Fig. 1E). Indeed, adenoviral overexpression of apobec-1, a cytidine deaminase and the catalytic component of the apoB mRNA-editing protein complex, leads to nearly complete disappearance of apoB100 and an increase of circulating apoB48 in both mice and rabbits. Furthermore, the synthesized and secreted VLDL particles were found to be smaller while LDL particles from the normal size range disappeared. Of note, the effect on total cholesterol levels in plasma was generally modest, with greatest reductions seen in adenovirus-treated LDL receptor deficient mice: 18% and 29% total cholesterol reduction in females and males, respectively. To our knowledge, no long-term experiments that employ the above strategy have been described to date.
Recently, other methods have been developed that inhibit protein synthesis by degrading specific mRNA sequences. One such strategy employs ribozymes, small RNA molecules with specific RNA cleaving capacity. Adenoviral expression of an apoB-specific ribozyme has been shown to decrease full-length apoB mRNA concentration and consequently the production of full-length apoB protein in vitro as well as in vivo (see Fig. 1F). Instead, a truncated variant of the protein was formed which was mainly degraded intracellularly. Inhibiting apoB100 protein synthesis with this adenoviral methodology lasted two weeks and led to a marked reduction of LDL-cholesterol but also TG levels in transgenic mice expressing human apoB and lacking hepatic apoB editing (Apobec1−/−ERhB−/− mice). More recently, Zhong et al. reported an AAV approach to express this ribozyme.51 Using AAV2, ribozyme production was evident for at least 150 days but expression levels were low and did not affect cholesterol levels. The recent description of double-stranded self-complementary (sc) AAVs, eliminating the need of second-strand synthesis which was shown to increase both early onset and level of expression52,53, prompted these authors to compare the expression levels of their original single-stranded vector with a sc-vector in HEPG2 cells. Compared to the original vector, the sc-vector was 3-fold more effective with regard to expression of the ribozymes. At day 3 this resulted in a 47% reduction of apoB100 mRNA levels while the single-stranded vector did not affect apoB100 mRNA levels.

Short-hairpin (sh) RNAs have also been employed to specifically knock down apoB production. In this context, self-inactivating lentivirus used for stably transfecting HEPG2 cells with apoB-specific shRNA, was shown to reduce both cellular and secreted apoB content by a marked 85% with persistence of this phenotype after 10 cell passages.54 Finally, a non-viral strategy using antisense oligonucleotides has recently been described which was shown to be very effective in reducing apoB100, but not apoB48, mRNA levels in mice.55 Specific 20-mer oligonucleotides were administered intraperitoneally, twice weekly for 6 weeks to C57/Bl6 wild-type mice, LDL receptor deficient mice and apoE deficient mice. Hepatic as well as intestinal apoB100 mRNA levels were reduced between 73% and 87% in all tested mouse models. These reductions were associated with a 25-55% decrease of plasma cholesterol, mainly caused by reductions in LDL-cholesterol levels but also in HDL-cholesterol. The apoE deficient mouse, however, only showed a decrease in the LDL-cholesterol fraction. Strikingly, 6 weeks after treatment was stopped, plasma cholesterol levels were still 38% decreased compared to pre-treatment levels (only tested in
the high-fat fed wild-type mice). A good safety profile in combination with the effectiveness in reducing LDL-cholesterol allowed this strategy to be tested in patients. Results of the first trial with this specific oligonucleotide, ISIS 301012, have recently been published. This study demonstrates a maximum reduction of apoB of 50% in the 2 highest dose treatment groups, i.e. 200 and 400 mg ISIS 301012. Coincided with this apoB decrease was a maximum 35% reduction in LDL-cholesterol, remaining decreased up to 3 months after the last dose. Given the efficacy of this compound, a phase II study has now been completed and results are anticipated.

**Increasing Cholesterol 7α-Hydroxylase**

The conversion of cholesterol to bile acids constitutes a major pathway by which cholesterol is eliminated from the body. Targeting this step in cholesterol metabolism, one group has studied the effects of increasing cholesterol 7α-hydroxylase expression since this is the rate-limiting enzyme in this process. To this purpose, wild-type hamsters were infected with adenovirus expressing cholesterol 7α-hydroxylase. In addition to enhanced conversion of cholesterol into bile and increased de novo cholesterol synthesis, these investigators noted a decrease in LDL secretion into the circulation but unaltered hepatic uptake of cholesterol from the circulation. This led to the hypothesis that hepatic up-regulation of cholesterol 7α-hydroxylase might also lead to decreased plasma LDL-cholesterol levels even in the absence of hepatic LDL receptors (see Fig. 1G). Indeed, adenoviral-mediated hepatic over-expression of cholesterol 7α-hydroxylase in LDL receptor deficient mice induced a dramatic decrease of plasma LDL-cholesterol levels, entirely due to decreased secretion from the liver.

**Concluding remarks on reducing low-density lipoproteins**

The current Hd-Ad, AAV and lentiviral vectors allow for persistent expression of LDL or VLDL receptors without evoking major immune responses in animal models for FH. Despite these promising data, no investigators are to our knowledge preparing for clinical application to treat FH in the near future. One of the reasons for this might be the challenge of pre-existing immunity against the viral vectors in the general population. For a recent example we wish to refer to a clinical trial that was described by Manno et al. The recent development of effective gene therapeutic means to degrade specific mRNAs offers new opportunities to treat FH by degrading apoB. Especially the non-viral antisense strategy
described by kastelein et al.\textsuperscript{56} is promising, despite the necessity of recurrent treatment.

**High-density lipoproteins**

HDL-cholesterol levels are inversely related to the risk of cardiovascular disease. How this lipoprotein exerts its beneficial effect is not entirely clear to date. In addition to its role in reverse cholesterol transport (RCT; often suggested to be key to the anti-atherogenic potential of HDL), HDL has recently been described to favorably affect endothelial function, attenuate inflammation, inhibit platelet aggregation and decrease apoptosis. The current quest to develop HDL-cholesterol increasing drugs is hampered by the notion that many of its key players need agonists to increase HDL-cholesterol levels. Here, gene therapy may offer a solution, however, as of yet no human studies have been carried out. In this respect, proof-of-principle may be obtained in subjects suffering from monogenetic disorders of HDL metabolism.

**Genetic causes of decreased high-density lipoprotein-cholesterol**

Mutations in three key proteins underlie deficits in HDL synthesis and maturation and thus its functioning to protect against atherosclerosis. Apolipoprotein A-I (apoA-I) is the principal protein component of HDL and besides being critically involved in RCT, has numerous other beneficial effects. Complete apoA-I deficiency is characterized by a near-complete absence of HDL and increased risk of premature coronary artery disease. Secondly, ATP binding cassette transporter A1 (ABCA1) deficient individuals who suffer from Tangier Disease, lack the capacity to lipidate nascent or essentially lipid-free apoA-I, thereby hampering HDL formation. Tangier disease patients are characterized by orange tonsils, cloudy corneas and intermittent peripheral neuropathy. With nearly absent HDL, these patients are at increased risk for atherosclerosis. Thirdly, lecithin-cholesterol acyltransferase (LCAT) deficient patients lack the capacity to esterify free cholesterol (delivered by ABCA1 action) on the surface of the HDL. This lack of cholesteryl ester synthesis in the circulation blocks maturation of nascent HDL into larger spherical HDL. Complete or partial LCAT deficiency (the latter is also known as Fish-eye Disease) is characterized by severe HDL deficiency, elevated TG levels and increased
risk of atherosclerosis. Gene therapeutic efforts for these three monogenetic disorders have all focused on introducing sufficient expression of wild-type copies of the respective genes.

**Apolipoprotein A-I overexpression**

ApoA-I gene therapy initially focussed on the question whether this strategy would result in proper secretion and incorporation of apoA-I into lipoprotein particles. Using adenoviral constructs it was shown that this was indeed the case but the level of apoA-I expression and distribution over the different lipoproteins appeared to be dependent of the mouse model of choice suggesting that the efficacy of apoA-I gene therapy might be dependent of the type of dyslipidemia of the recipient mouse. Subsequent development of a gene therapeutic approach for apoA-I delivery focused on enhancing both expression as well as persistence of expression. Using various constructs these investigators evaluated the effects of 1) liver-specific promoters vs. the ubiquitously active CMV promoter, 2) genomic DNA (including introns) vs. complementary DNA encoding for apoA-I, and 3) inclusion of enhancers (changing copy number and upstream/downstream position). It was shown that liver-specific promoters induced prolonged expression compared to the CMV promoter largely due to less transgene expression in antigen-presenting cells, reduced hepatotoxicity and lack of promoter-silencing. In addition, the introduction of introns in genomic DNA constructs enhanced transgene expression compared to the use of apoA-I cDNA. Finally, inclusion of up to four copies of the apoE-enhancer increased levels of expression significantly, with the notion that enhancers in a 5’-position increased hepatotoxicity and consequently reduced duration of expression. Eventually an adenoviral vector was constructed with the liver-specific human α1-antitrypsin promoter, genomic DNA encoding apoA-I and 4 copies of the apoE enhancer in the 3’-position. This vector was shown to increase plasma levels of human apoA-I to a maximum of 500 mg/dL, i.e. 3-4 times higher than human physiological levels (C57/Bl6 mice; 10-12 days after gene transfer). The expression was lost after four months indicating that promoter silencing, hepatotoxicity and immune responses were overcome in part. Loss of transgene expression at this point was ascribed to residual low-level hepatotoxicity of the viral proteins present in the adenoviral vector. Eliminating the majority of these viral proteins using a helper-independent E1/E3/E4-deleted adenovirus avoided such hepatotoxicity and resulted in persistent expression beyond 120 days.
However, this came with a reduction of peak apoA-I expression levels (200 mg/dL; still well above physiological levels).66 More recently, other viral vectors have been employed to drive long-term apoA-I expression; Hd-Ad vectors, lacking all viral proteins, were shown to boost apoA-I levels up to 200 mg/dL for at least 24 weeks with minimal hepatotoxicity.67,68 This expression level of human apoA-I on top of endogenous murine apoA-I resulted in a significant increase in HDL-cholesterol levels and a significant reduction of the atherosclerotic burden between 30 and 50% in both LDL receptor deficient67 as well as apoE deficient mice.68 More recently, Oka et al. demonstrated in apoA-I deficient mice proof of principle that this approach of liver-directed Hd-Ad gene delivery is effective for long-term phenotypic correction of monogenic hypoalphalipoproteinemia.69

In 2005, Sharifi et al. used AAV to mediate apoA-I gene transfer in apoA-I deficient mice.70 An intriguing feature of this study was the use of a naturally occurring apoA-I variant, i.e. apoA-I-Milano, instead of wild-type apoA-I as the transgene. In apoA-I-Milano an Arg residue is substituted for a Cys residue at position 173. In individuals carrying this mutation, apoA-I-Milano is reported to be catabolized at an increased rate thereby increasing the hepatic uptake of tissue lipids and conferring greater resistance against atherosclerosis.71 Comparing AAV serotypes 1, 2 and 5, Sharifi et al.70 showed that AAV1 yielded highest expression both after intravenous (IV) as well as after intramuscular (IM) administration. Both administration routes showed highest apoA-I expression levels at 24 weeks when the animals were sacrificed. This is remarkable when considering the use of a CMV promoter that is known to suffer from silencing. With IV injections performing best with expression up to 25 mg/dL apoA-I, this is still well below apoA-I levels of wild-type mice, typically ranging from 60 to 100 mg/dL. This result may be explained by an enhanced catabolic rate of apoA-I-Milano compared to wild-type apoA-I but more likely reflects the use of a suboptimal AAV serotype. Evidence for this idea comes from studies by Kitajima et al. who compared AAV2 with novel AAV serotypes 7 and 8.72 Whereas AAV2 encoding human apoA-I and driven by a liver-specific promoter resulted in a peak expression of only 6 mg/dL after IV injection, the use of AAV7 or AAV8 increased expression up to 143 and 69 mg/dL, respectively with both serotypes also causing significant increases in HDL-cholesterol levels in C57/Bl6 wild-type mice. Furthermore, expression was persistent for up to one year.

Besides investigating the extent and duration of expression to eventually realize clinical use of apoA-I gene therapy, somatic gene transfer of apoA-I
has also been used to better understand the role of apoA-I in lipid metabolism and beyond. This includes e.g. studies into the effect of apoA-I expression on neo-intima formation after endothelial denudation\textsuperscript{73}, vasoactive properties of HDL\textsuperscript{74}, HDL-associated proteins such as platelet-activating factor acetylhydrolase and paraoxonase 1\textsuperscript{75}, HDL assembly\textsuperscript{76}, RCT from macrophages to feces\textsuperscript{77} and atherogenesis.\textsuperscript{67,68,78,79}

**LCAT overexpression**

Few efforts have been made to develop a gene therapeutic strategy for treating LCAT deficiency, a disorder which can cause early death due to renal failure.\textsuperscript{80} In a first study, Seguret-Mace et al. used first generation adenoviral constructs for LCAT gene delivery in human apoA-I transgenic mice.\textsuperscript{81} These investigators showed a strong increase in plasma LCAT activity and a significant increase in HDL-cholesterol with a concomitant rise in human apoA-I. Furthermore, HDL particles increased in size suggesting efficient maturation. Using adenovirus as a vector in mice lacking leptin as well as the LDL receptor, Mertens et al. subsequently showed that increased levels of human LCAT reduced the atherosclerotic burden.\textsuperscript{82} Intriguingly, the increase of LCAT activity in the latter experiment did not result in changes of HDL-cholesterol levels or cholesteryl ester to free cholesterol ratio in plasma. Instead, the observed protection from atherosclerosis was ascribed to the prevention of LDL oxidation caused by the increased LCAT activity.

Finally, Fan et al. tested a strategy in which LCAT and its principal activator apoA-I were simultaneously expressed.\textsuperscript{83,84} Using either AAV or retroviral vectors they showed that both transgenes could be expressed and secreted from a single vector in hepatocytes and myoblasts in vitro. Although a very interesting concept, functional efficacy and evaluation in vivo have not been described.

**ABCA1 overexpression**

Wellington et al. are the only investigators who have investigated the use of gene therapy to achieve overexpression of ABCA1.\textsuperscript{85} To evaluate whether hepatic overexpression could affect plasma HDL-cholesterol levels, C57/Bl6 wild-type mice were treated with an adenoviral vector in which the expression of human \textit{ABCA1} cDNA was driven by the CMV promoter. HDL-cholesterol proved to increase dose-dependently when using viral dosages up to $5 \times 10^8$ pfu. Higher dosages, however, did not
Review gene therapy and lipid disorders

Further increase HDL-cholesterol but did, unexpectedly, dose-dependently increase total cholesterol, triglycerides, phospholipids and apoB levels in plasma. These data suggest that markedly increased expression of ABCA1 alters whole body lipid homeostasis and the authors suggested that therapeutic application can only be pursued if transgenic ABCA1 expression can be placed under physiological regulation.

Concluding remarks on increasing high-density lipoproteins

Despite many efforts to achieve therapeutic correction of low HDL-cholesterol levels, clinical trials are not planned in the near future. There may be a future for apoA-I gene therapy since this protein has multiple favorable characteristics which may drive more investigators to optimize gene delivery and expression of this protein. However, it is a challenging target since one needs very high expression levels in humans to achieve therapeutic impact. For LCAT, there exists little evidence that overexpression is indeed anti-atherogenic and, as for apoA-I deficiency and Tangier disease, it will be difficult to recruit sufficient numbers of patients for initial trials. When gene therapy is shown to be safe and efficacious, the use of apoA-I, LCAT or ABCA1 as transgenes in patients at very high risk of cardiovascular disease may be possible.

Triglycerides

Genetic causes of elevated triglyceride levels

Hypertriglyceridemia can result from overproduction of triglyceride-rich lipoproteins or from attenuated hydrolysis and subsequent clearance of these lipoproteins. This lipid disorder is often the result from a detrimental interaction between genetic and environmental/demographic factors. Overproduction of TG-rich lipoproteins in various genetically transmitted dyslipidemias, among others FCH, has in general an unknown etiology. However, impaired TG hydrolysis is in some cases caused by well-defined defects in key enzymes involved in this process. Homozygosity or compound heterozygosity for mutations in lipoprotein lipase (LPL), the critical enzyme of TG hydrolysis in the circulation, leads to massive elevation of plasma TG levels, also known as chylomicronemia (accumulation of TG-containing chylomicrons in the circulation). Mutations in the primary cofactor of LPL, i.e. apolipoprotein C-II (apoC-II) cause a
nearly identical phenotype. More recently, defects in apoA-V have also been shown to cause hypertriglyceridemia but often in combination with increased total cholesterol levels.

Regarding gene therapeutic efforts to treat hypertriglyceridemia, LPL deficiency has been the main focus. Several factors make this rare genetic disorder an interesting target. First of all, LPL deficiency is a potentially life-threatening disorder that in general is associated with a low quality of life. Current treatment strategies are often insufficient to prevent hospitalization due to acute haemorrhagic pancreatitis. Importantly, the majority of patients suffer from LPL mutations that result in loss of catalytic activity but the mutated protein is still produced and secreted into the circulation. Hence, transgenic LPL is less likely to induce an immune response. Finally, this disorder can be phenotypically corrected by only partially restoring LPL activity levels.

**Increasing lipoprotein lipase**

In 1995 the first in vitro studies demonstrated that inducing LPL expression resulted in proper secretion of catalytically active LPL by a variety of cells. This fits with the natural expression of LPL in many tissues in early development. In adulthood, however, LPL is only expressed in skeletal muscle, cardiac muscle, adipose tissue and macrophages. Using adenoviral vectors, Excoffon et al. showed that ectopic expression of LPL in the liver of heterozygous LPL deficient mice resulted in the secretion of catalytically active LPL which caused a decrease in predominantly VLDL-TG. Importantly, the transgenic LPL improved post-prandial TG clearance when the treated mice were challenged with an oral fat load. However, the hepatotoxic nature of this early adenoviral vector resulted in negligible LPL mRNA levels in the liver at 42 days after gene transfer. Further studies with adenovirus demonstrated that LPL overexpression could also alleviate hyperlipidemias of a different nature. In apoE deficient mice and LDL receptor deficient mice LPL gene therapy was shown to result in marked reductions in plasma TG levels (63% and 40%, respectively) and total plasma cholesterol levels (48% and 43%, respectively). Using the same approach, Liu et al. even showed that TG hydrolysis could be enhanced in wild-type mice. An important step toward human application was a proof-of-principle study in a large, natural-occurring animal model; the LPL deficient cat. Using the same adenoviral vector in this model, it was shown that ectopic LPL expression caused a striking 90% reduction of plasma TG levels but only for up to 2
weeks. An antibody response against both viral proteins as well as the human LPL protein explained this transient expression. The latter is probably related to the fact that these animals are homozygous for a null-mutation for LPL in which case the introduced human LPL protein acted as a neo-antigen. Despite this drawback, this study demonstrated that phenotypic correction of hypertriglyceridemia due to complete LPL deficiency could be efficiently corrected using somatic gene transfer. On the other hand, vectors that could mediate long-term expression were needed. A murine model of complete LPL deficiency was subsequently generated to further develop LPL gene therapy. In pursuit of long-lasting transgene expression, these investigators switched to IM administration of AAV serotype 1 to express LPL$^{S447X}$. Compared to the wild-type protein, LPL$^{S447X}$ has been shown to be associated with decreased plasma TG, increased HDL-cholesterol, decreased LDL-cholesterol and significantly reduced risk of coronary artery disease (for review see). Expression of LPL$^{S447X}$ was thus reasoned to be superior to expression of LPL$^{WT}$, as a means to reduce TG levels. Using this approach, partial restoration of LPL activity levels (33%) resulted in a near-complete normalization of plasma TG, TC, HDL-cholesterol and free fatty acid levels for over one year. Moreover, the TG clearance after an intravenous fat load, impaired in LPL deficient mice, was greatly improved upon treatment with LPL$^{S447X}$. This very strategy also proved effective in the LPL deficient cat but again this effect was only transient (2 weeks): similar to using Ad-LPL, development of neutralizing antibodies was observed. Combined with safety and biodistribution studies, these proof-of-principle studies allowed for the initiation of a first clinical trial. This trial started in October 2005 and focused on safety and efficacy of the IM application of AAV1-LPL$^{S447X}$ in LPL deficient patients. All these patients have low to normal levels of catalytically inactive LPL in the circulation. The presence of this mutated protein is anticipated to reduce chances on the development of an immune response of the host against the transgene product. Currently 7 out of 8 participants have been included and results of this trial are anticipated in the near future.

**ApoA-V overexpression**

Since the discovery of apoA-V in 2001, 3 premature truncation mutations have been described that in combination with secondary causes, e.g. pregnancy, age and obesity, lead to massive elevation of plasma TG levels in man. Although not directly aimed at developing a therapy,
several investigators have shown that adenoviral vectors can be successfully used to induce overexpression of apoA-V which is naturally expressed in the liver. Treating apoA-V deficient mice, which have severely increased plasma TG levels, with an adenovirus expressing human apoA-V resulted in a marked 70% decrease in TG levels and a cholesterol-lowering in all lipoprotein classes but especially in HDL.\textsuperscript{103} Using the same adenoviral approach in wild-type C57/Bl6 mice, the TG-lowering effect of apoA-V overexpression was shown to be predominantly caused by activation of LPL-mediated TG hydrolysis and reducing the hepatic VLDL-TG production rate.\textsuperscript{104} Recently, Huang et al.\textsuperscript{105} demonstrated that adenoviral-mediated expression of apoA-V in apoE deficient mice, also results in strong decreases of plasma cholesterol and triglyceride levels by 58 and 75%, respectively, at day 7 after gene transfer. Remarkably, however, in these apoE deficient mice HDL-cholesterol levels remained unchanged.

**Concluding remarks on decreasing triglycerides**

The first clinical gene therapy trial to increase LPL, using IM application of AAV serotype 1, is currently under way. If this strategy proves safe and efficacious, it may set the stage for gene therapy to treat other monogenetic disorders of lipid metabolism as described in this review. In addition, such therapy may be applicable for other inherited diseases for which there exists no or insufficient medication to date.

**Future direction and concluding remarks**

With a history of two decades and one landmark study in humans\textsuperscript{21}, gene therapeutic approaches in treating dyslipidemia are still maturing. This actually holds true for the entire gene therapy field, mainly because the current viral vectors are still not ideal. Although Hd-Ad, lentiviral and AAV vectors are promising in terms of expression levels and toxicity, immune responses against the viral capsid proteins hamper their clinical application. Indeed, exposure to these viral vectors invariably leads to the mount of an adaptive immune response. This not only prevents repeated administration, but also poses an initial problem since a large part of the general population has a pre-existing immune response due to earlier encounters with the natural variants of these viral vectors. Recent developments are therefore aimed at circumventing pre-existing immune
responses for example by using vectors with coating proteins that are not recognized by the host such as different viral serotypes from other species that are not present in humans\textsuperscript{106-108}, hybrid vectors\textsuperscript{108} or viral particles with masked capsid proteins.\textsuperscript{109} Other investigators focus on antigen-presenting cells such as macrophages which can function as the initiators of adaptive immune responses. Modulation can be achieved for example by immune-suppressive regimes\textsuperscript{110-112}, temporarily eliminating these antigen-presenting cells for example using dichloro-methylene-biphosphonate\textsuperscript{113} or by expressing immuno-suppressant sequences, e.g. g019, to prevent presentation to the viral MHC components.\textsuperscript{114} Given the ongoing development of better-equipped vectors, especially with respect to avoiding immune responses, it appears that clinical application is at the horizon. For the lipid research field, gene therapy is likely to be tested in various cohorts with monogenetic lipid disorders. Only if these trials proof to be safe and efficacious, broader application for patients at very high risk of cardiovascular disease may be possible.

References


Review gene therapy and lipid disorders


of low-density lipoprotein receptors to hyperlipidemic rabbits: receptor expression modulates high-density lipoproteins. *Metabolism*: 1996;45: 1447-1457.


Review gene therapy and lipid disorders


Review gene therapy and lipid disorders


Review gene therapy and lipid disorders


Review gene therapy and lipid disorders


