Review
Gene therapy for lipid disorders

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Abstract

Lipid disorders are associated with atherosclerotic vascular disease, and therapy is associated with a substantial reduction in cardiovascular events. Current approaches to the treatment of lipid disorders are ineffective in a substantial number of patients. New therapies for refractory hypercholesterolemia, severe hypertriglyceridemia, and low levels of high-density lipoprotein cholesterol are needed: somatic gene therapy is one viable approach. The molecular etiology and pathophysiology of most of the candidate diseases are well understood. Animal models exist for the diseases and in many cases preclinical proof-of-principle studies have already been performed. There has been progress in the development of vectors that provide long-term gene expression. New clinical gene therapy trials for lipid disorders are likely to be initiated within the next few years.

Keywords: atherosclerosis, cholesterol, gene therapy, lipoproteins, vectors

Introduction

A reduction in plasma cholesterol levels is associated with a substantial reduction in cardiovascular events [1]. Methods for reducing cholesterol levels and specifically low-density lipoprotein (LDL) cholesterol include lifestyle changes and drug therapy, including statins, niacin, bile acid sequestrants, and fibrates [2]. However, many patients cannot achieve optimal LDL cholesterol levels with existing cholesterol-lowering therapies [2]. The majority of these patients have genetic disorders that cause severe hypercholesterolemia that is partly refractory to existing therapies, whereas other patients do not tolerate existing therapies. Hypercholesterolemia therefore remains an important target for the development of novel therapies.

Plasma triglycerides are increasingly recognized as a risk factor for cardiovascular disease [3], and severe hypertriglyceridemia is associated with acute pancreatitis. Although triglyceride levels can frequently be decreased with a combination of lifestyle changes and drug therapy such as fibrates and niacin, there remain a substantial number of individuals in which triglycerides remain significantly elevated. Hypertriglyceridemia is therefore also a target for the development of novel therapies.

AAV = adeno-associated virus; ABC1 = ATP-binding cassette protein 1; apoA-I = apolipoprotein A-I; apoE = apolipoprotein E; CETP = cholesteryl ester transfer protein; FH = familial hypercholesterolemia; HDL = high-density lipoprotein; LAL = lysosomal acid lipase; LCAT = lecithin:cholesterol acyltransferase; LDL = low-density lipoprotein; LPL = lipoprotein lipase; MTP = microsomal transfer protein; VLDL = very-low-density lipoprotein; WHHL = Watanabe heritable hyperlipidemic.

Finally, low plasma levels of high-density lipoprotein (HDL) cholesterol are associated with significantly increased cardiovascular risk [4]. Low-HDL cholesterol is one of the most common lipid disorders in patients with premature coronary artery disease [5]. There are a variety of genetic causes of low-HDL cholesterol [6]. A variety of data from animals and from humans suggest that increasing the HDL cholesterol concentration is beneficial. However, the majority of patients have difficulty raising the HDL cholesterol level despite lifestyle changes and drug therapy such as niacin and fibrates. Low-HDL cholesterol is therefore a very important target for the development of novel therapies.

There is much interest in the development of new small molecules that decrease LDL cholesterol, decrease triglycerides, or raise HDL cholesterol levels. In addition, gene therapy has been considered as a potential approach to some of these targets. The purpose of this article is to review the specific lipid disorders that are potential candidates for the development of clinical gene therapy, to discuss the specific issues involved for these disorders, to summarize the proof-of-principle studies in animals where such studies exist, and to comment on the potential progress toward clinical trials of gene therapy for these disorders.

Lipid disorders that are candidates for gene therapy

Disorders associated primarily with hypercholesterolemia or hypertriglyceridemia

Familial hypercholesterolemia
Familial hypercholesterolemia (FH) is caused by mutations in the LDL receptor [7]. Homozygous FH, owing to mutations in both LDL receptor alleles, occurs in about 1 in one million individuals worldwide and is associated with markedly elevated LDL cholesterol levels (about 500–1000 mg/dl) and markedly premature atherosclerotic cardiovascular disease. FH homozygotes are highly resistant to currently available drug therapy. The preferred treatment at present is LDL apheresis, a physical procedure in which LDL is selectively removed from the blood by passing plasma over columns that bind the LDL [8]. LDL apheresis must be performed every 1–2 weeks, and although patients on regular apheresis seem to have delayed onset and progression of atherosclerotic cardiovascular disease, they remain at a much higher than average risk. For these reasons, homozygous FH is a candidate for the development of gene therapy.

Although LDL receptors are expressed ubiquitously, the hepatic LDL receptor has the greatest quantitative effect in controlling plasma LDL levels. Importantly, a limited number of patients with homozygous FH have undergone liver transplantation and have experienced substantial reductions in LDL cholesterol levels. This indicates that gene therapy targeted toward the liver would be likely to be an effective therapy for this disease. The number of hepatocytes and the level of gene expression necessary to result in a therapeutic effect are uncertain. However, it is worth noting that patients with homozygous FH are frequently classified as either receptor-negative (no detectable functional LDL receptor activity) or receptor-defective (markedly reduced but detectable LDL receptor activity). Receptor-negative FH is associated with significantly higher cholesterol levels and earlier onset and severity of atherosclerotic cardiovascular disease than receptor-defective FH. A gene therapeutic approach that resulted in enough LDL receptor expression to convert a receptor-negative patient to 'receptor-defective' could therefore be considered therapeutically beneficial.

A considerable number of proof-of-principle studies have been performed in animal models of homozygous FH. The Watanabe heritable hyperlipidemic (WHHL) rabbit is homozygous for a natural mutation in the LDL receptor. A recombinant retrovirus was used to achieve *ex vivo* gene transfer of the rabbit LDL receptor to hepatocytes from WHHL rabbits, with subsequent reinfusion of the genetically modified hepatocytes. Plasma cholesterol levels were reduced by about 30% and persisted for 6 months [9]. Injection of a recombinant first-generation adenovirus encoding the LDL receptor into WHHL rabbits transiently reduced LDL cholesterol levels [10,11]. The LDL receptor knockout mouse has also been used as an FH model. The administration of a first-generation adenovirus encoding the human LDL receptor transiently reduced LDL cholesterol levels in chow-fed mice deficient in LDL receptor [12] and reduced cholesterol levels and atherosclerosis in mice deficient in LDL receptor that were fed with a diet high in fat (M Kawashiri, DJ Rader, unpublished data). Administration of antibody against CD40 ligand at the time of administration on recombinant LDL receptor adenovirus decreased immune-mediated promoter silencing and resulted in long-term transgene expression and cholesterol decrease in mice deficient in LDL receptor [13].

Although the LDL receptor is the logical gene to use for gene therapy of FH, there is concern that the LDL receptor expressed as a result of somatic gene transfer could be immunogenic in patients that lack the ability to synthesize the LDL receptor. Other genes have therefore been used in animals in attempts to ameliorate the hypercholesterolemia associated with a deficiency in LDL receptors. The very-low-density lipoprotein (VLDL) receptor is a lipoprotein receptor with homology to the LDL receptor, but it is not normally expressed in the liver. The murine VLDL receptor was expressed in the livers of mice deficient in LDL receptor by using a first-generation adenovirus; LDL cholesterol levels were reduced to a comparable degree and for a longer period compared with the expression of the LDL receptor [14]. A recombinant adeno-associated virus (AAV) was used to express the murine VLDL receptor in

the livers of mice deficient in LDL receptor fed on a high-fat Western diet. Expression of the VLDL receptor decreased plasma cholesterol levels significantly for more than 7 months and reduced the development and progression of atherosclerosis [15]. Another novel approach is the use of chimeric molecules made by fusion of a soluble form of the LDL receptor with transferrin [16] that could potentially bind LDL in the circulation and mediate clearance through the transferrin receptor on hepatocytes.

Homozygous FH is the only lipid disorder that has been approached in a clinical gene therapy trial [17]. The trial used an *ex vivo* approach in which five homozygous FH patients underwent a surgical resection of the left lateral segment of the liver. Primary hepatocytes were isolated and after 2 days were infected with a recombinant retrovirus encoding the human LDL receptor gene. The genetically modified hepatocytes were harvested 24 h later and reinfused into the patients via a portal catheter that was placed in the inferior mesenteric vein at the time of the original surgery. Patients tolerated the infusions of autologous hepatocytes well without complications. Two of five patients had a decrease of about 20% in their LDL cholesterol levels. Kinetic studies of LDL metabolism demonstrated that LDL catabolism was increased in the same two patients, which was consistent with increased LDL receptor expression. Liver biopsies performed 4 months after treatment revealed LDL receptor transgene expression by *in situ* hybridization in all five subjects.

Homozygous FH will continue to be a model for the development of liver-directed somatic gene therapy. However, many heterozygous FH patients are also relatively refractory to existing drug therapy and remain at very high risk for development and progression of atherosclerotic vascular disease. Therefore, once a gene therapeutic strategy is found to be effective in homozygous FH, it might be extended to clinical trials in severe heterozygous FH as well.

Lipoprotein lipase deficiency (familial hyperchylomicronemia)
Lipoprotein lipase (LPL) deficiency is a rare autosomal recessive disorder characterized by markedly elevated plasma levels of triglycerides [18]. Normally, triglycerides in chylomicrons are hydrolyzed by LPL, and the genetic absence of LPL results in marked hyperchylomicronemia. LPL deficiency is associated with recurrent episodes of acute pancreatitis, which can be life-threatening and can also lead to chronic pancreatic insufficiency. LPL deficiency is often refractory to diet and drug therapy and is therefore a candidate for the development of gene therapy.

Several animal models of LPL deficiency exist. LPL knockout mice have severe hypertriglyceridemia and die within 24 h of birth [19]. Germline transgenic expression of LPL in skeletal muscle rescued the mice and normalized the triglyceride levels [20]. Cats and minks that are naturally

deficient in LPL have been reported [21,22]. Liver-directed overexpression of human LPL with an adenoviral vector in LPL-deficient cats reduced triglyceride levels for up to 14 days [23].

Most LPL expression is in muscle and adipose tissue. Because skeletal muscle is an attractive target for somatic gene transfer, LPL deficiency is a candidate for the development of muscle-directed gene therapy. The intramuscular injection of recombinant AAV vectors in mice resulted in the stable expression of several secreted proteins [24–26]. Correction of hemophilia B was achieved by the intramuscular injection of an AAV vector in dogs [27] and in humans [28]. AAV might therefore be an attractive vector for the use of muscle-directed LPL gene transfer. The major issues are whether enough LPL expression could be achieved to normalize triglyceride levels and the duration of gene expression.

Gene transfer to increase LPL expression could potentially have therapeutic value in conditions other than homozygous LPL deficiency. Transgenic mice overexpressing LPL have less diet-induced hyperlipidemia, less hyperlipidemia associated with diabetes, and reduced diet-induced atherosclerosis. Liver-directed gene transfer of LPL with recombinant adenovirus decreased fasting and post-prandial levels of triglycerides in heterozygous LPL-deficient mice [29] and decreased levels of triglycerides and cholesterol in mice deficient in apolipoprotein E (apoE) and in LDL receptor [30].

ApoE deficiency and familial dysbetalipoproteinemia
ApoE serves as a ligand that mediates the clearance of chylomicron and VLDL remnant lipoproteins by binding to the LDL receptor and related members of the same gene family [31]. Most apoE in plasma is derived from the liver. A genetic deficiency of apoE results in substantially elevated levels of lipoprotein remnants and is associated with an increased risk for atherosclerotic vascular disease [32]. ApoE knockout mice develop high levels of remnant lipoproteins and severe atherosclerosis on a chow diet [33]. Liver-directed gene transfer of human apoE markedly decreased plasma cholesterol levels for about 2 weeks with a first-generation adenovirus [34,35] and for at least 8 weeks with a second-generation adenovirus [36]. Gene transfer of apoE to the liver in apoE-deficient mice also induced substantial regression of atherosclerosis [37,38]. Although plasma apoE is mostly derived from the liver, apoE produced in any tissue could theoretically be effective if secreted into the blood. ApoE derived from macrophages after transplantation of wild-type bone marrow into apoE-deficient mice also normalized plasma cholesterol levels and reduced the development of atherosclerosis [39]. ApoE gene therapy in patients with apoE deficiency would correct the hyperlipidemia but this condition is extremely rare and is therefore not an optimal model for the development of this type of gene therapy.

ApoE gene therapy could have therapeutic value in another apoE-related lipid disorder sometimes called familial dysbetalipoproteinemia or Type III hyperlipoproteinemia [40]. In this disorder, a mutant apoE has decreased binding to receptors, thus resulting in a reduced clearance of remnant lipoproteins and hyperlipidemia. The most common cause is homozygosity for a common polymorphism in apoE called apoE2 (the wild-type form is known as apoE3). Rarer causes are other apoE mutations that often cause hyperlipidemia in a dominant fashion. Gene therapy to increase the hepatic production of wild-type apoE3 could be a therapeutic strategy for this condition.

Familial combined hyperlipidemia and diabetic dyslipidemia
Familial combined hyperlipidemia is an inherited phenotype characterized by elevated VLDL and LDL; it is believed to be normally due to overproduction of VLDL by the liver [41]. The specific genetic basis of this common disorder is currently unknown. Type II diabetes mellitus is also characterized by the hepatic overproduction of VLDL and increased VLDL and LDL cholesterol [42]. Both of these conditions are associated with a significantly increased risk for atherosclerotic vascular disease. Although many patients with these disorders can be adequately treated with lifestyle and drug therapy, a substantial number cannot achieve adequate control of their hyperlipidemia. Gene therapy might therefore be one therapeutic approach to this class of patients with refractory combined hyperlipidemia.

One approach could be expression of the VLDL receptor in the liver. As noted above, although the VLDL receptor is not normally expressed in the liver, it could theoretically bind VLDL and reduce the levels of both VLDL and LDL. Proof-of-principle studies in relevant animal models are needed to address this question. Another approach could be increasing the hepatic expression of apoE. Because apoE serves as a ligand for the LDL receptor, other related receptors, and heparan sulfate proteogycans in the liver, increased apoE expression could potentially target more VLDL and remnants for hepatic degradation, thus reducing VLDL and LDL levels. Again, proof-of-principle studies in relevant animal models are needed to address this question. Specifically, more data are needed on the effects of hepatic apoE gene transfer on lipoprotein metabolism in dyslipidemic animal models other than apoE deficiency.

Disorders associated primarily with low-HDL cholesterol
Lecithin : cholesterol acyltransferase deficiency
Unesterified cholesterol is esterified to cholesteryl ester in the blood by the lipoprotein-associated enzyme lecithin : cholesterol acyltransferase (LCAT). Complete LCAT deficiency is is characterized by markedly reduced HDL cholesterol levels (less than 10 mg/dl), corneal opacities, anemia, and progressive proteinuria and renal insufficiency

eventually leading to end-stage renal disease [43]. Several different mutations in the LCAT gene have been described in patients with LCAT deficiency. LCAT knockout mice also have reduced HDL cholesterol levels [44], although the development of renal disease has not been reported. Because this disorder has no known therapy to prevent the progressive renal disease, it is a candidate for the development of gene therapy. Although LCAT is normally synthesized in the liver, because it is a secreted protein it could theoretically be made in other tissues such as muscle after gene transfer.

Tangier disease
Tangier disease is a rare genetic disorder associated with markedly reduced HDL cholesterol levels (less than 5 mg/dl), the accumulation of cholesterol in macrophages and related cells, neuropathy, and premature atherosclerosis [45]. It is caused by mutations in the ATP-binding cassette protein 1 (ABC1), a cellular protein that promotes the efflux of cellular cholesterol and phospholipids to nascent HDL and lipid-poor apolipoprotein A-I (apoA-I) [46]. ABC1 knockout mice also have markedly reduced HDL cholesterol levels and cholesterol storage in macrophages and macrophage-like cells [47]. A naturally occurring model of Tangier disease in chickens has been reported [48]. Gene transfer of ABC1 to macrophages or bone marrow stem cells would be likely to ameliorate the symptoms of this disorder, but no proof-of-principle studies have yet been reported in animals.

Importantly, heterozygotes for ABC1 mutations have significantly reduced HDL cholesterol levels (less than 10th percentile) [49] and are at increased risk for atherosclerotic vascular disease [50]. There are no drug therapies that raise HDL cholesterol levels in these patients. Macrophage-directed gene therapy with ABC1 could therefore be an approach to this disorder as well.

ApoA-I deficiency
ApoA-I is the major protein in HDL; a genetic deficiency of apoA-I results in markedly reduced HDL and seems, at least in some kindreds, to increase the risk for coronary artery disease [51]. ApoA-I knockout mice have extremely low levels of HDL cholesterol and although chow-fed mice do not develop atherosclerosis [52], the absence of apoA-I enhances the progression of atheroclerosis in hyperlipidemic mouse models [53,54]. The expression of human apoA-I in apoA-I knockout mice by using a first-generation adenovirus transiently restored HDL cholesterol levels to the normal range [55]. In apoA-I-deficient patients, gene transfer of apoA-I to liver (or possibly muscle) would be expected to increase HDL cholesterol levels and over time would reduce the risk for atherosclerotic disease. However, apoA-I-deficient patients are very rare, making this disease somewhat less attractive as a target for the development of gene therapy.

Other disorders of reduced HDL cholesterol
Reduced levels of HDL cholesterol are common, and most patients with low HDL do not have mutations in LCAT, ABC1, or apoA-I. However, it is possible that gene transfer to express any of these genes could nevertheless be therapeutically useful. For example, by converting unesterified to esterified cholesterol, LCAT is believed to facilitate the process of 'reverse cholesterol transport'. Transgenic over-expression of human LCAT increased HDL cholesterol levels in mice [56–58] and rabbits [59,60]. Administration of a first-generation adenovirus encoding human LCAT in human apoA-I transgenic mice resulted in a substantial increase in HDL cholesterol and human apoA-I levels [61]. Although transgenic LCAT overexpression increased atherosclerosis in cholesterol-fed mice [62], co-expression with cholesteryl ester transfer protein (CETP) reduced atherosclerosis in mice [63]. Furthermore, transgenic expression of LCAT in cholesterol-fed rabbits reduced atherosclerosis [64]. Thus, LCAT might be a candidate for gene therapy to inhibit atherosclerosis in humans. Similarly, gene therapy to augment ABC1 expression in macrophages might be expected to reduce atherosclerosis even in patients who do not have ABC1 mutations. Animal experiments are needed to test this hypothesis.

Perhaps the best evidence for this concept exists for apoA-I. A variety of evidence in animals indicates that intervention to increase the expression of apoA-I is likely to be associated with reduced atherosclerosis. Weekly intravenous injection of human HDL into cholesterol-fed rabbits resulted in the regression of atherosclerotic lesions [65] and regular intravenous injection of rabbit apoA-I into cholesterol-fed rabbits resulted in the reduced progression of atherosclerotic lesions [66]. Hepatic over-expression of human apoA-I in trangenic mice reduced atherosclerosis in C57BL/6 mice fed with a high-fat diet [67] and in apoE-deficient mice [68,69]. Transgenic over-expression of human apoA-I in the livers of hyperlipidemic WHHL rabbits reduced the development of aortic atherosclerosis [70]. Gene transfer of human apoA-I with a first-generation adenoviral vector in human apoA-I-transgenic/apoE-deficient mice reduced the progression of atherosclerosis [71]. Administration of a second-generation adenovirus resulted in the significant regression of pre-existing atherosclerotic lesions in mice deficient in low-density lipoprotein receptor that were fed with a Western diet [72]. These results indicate the feasibility of apoA-I gene transfer as a strategy for affecting atherosclerosis. ApoE is another candidate for gene transfer and overexpression as a strategy to reduce atherosclerosis [73].

Other disorders associated with defects in lipid and lipoprotein metabolism
Wolman disease and cholesteryl ester storage disease
Lysosomal acid lipase (LAL) is required for the hydrolysis of lipoprotein-derived cholesteryl esters and triglycerides in the lysosome. Wolman disease and cholesteryl ester storage disease are autosomal recessive disorders caused by mutations in LAL. Wolman disease is the rare and more severe form in which infants develop hepatosplenomegaly, steatorrhea, adrenal calcification, failure to thrive, and early death by the second year of life. Patients with cholesteryl ester storage disease have hepatomegaly and hyperlipidemia in childhood and often progress to fibrotic liver disease. The reason for the differences in phenotype might be related to the specific molecular defects underlying the LAL deficiency [74]. A natural rat model of this disorder [75] and the acid lipase knockout mouse [76] each have many of the pathologic features of the human disease. Correction of the LAL deficiency in fibroblasts obtained from Wolman disease patients by using a recombinant adenovirus reduced the lipid storage and increased cell growth [77]. LAL deficiency disorders are attractive for gene therapy because the restoration of a relatively small amount of LAL expression in the liver would be expected to ameliorate the disease.

Abetalipoproteinemia
The microsomal transfer protein (MTP) mediates the microsomal transport of lipids in the intestine and liver and is necessary for the normal formation and secretion of chylomicrons in the enterocyte and VLDL in the hepatocyte [78]. Abetalipoproteinemia is a rare autosomal recessive disease caused by mutations in MTP. Abetalipoproteinemia is characterized by progressive spinocerebellar degeneration and pigmented degenerative retinopathy, leading to severe ataxia, blindness, and eventually premature death. These symptoms are the result of defects in the absorption and transport of fat-soluble vitamins, especially vitamin E, which requires the VLDL–LDL system for adequate transport to peripheral tissues. Therapy with high-dose vitamin E and other fat-soluble vitamins can slow but not prevent the progression of these symptoms. Knockout of MTP is lethal during embryogenesis [79] but the MTP gene was deleted in the liver after birth, creating a mouse model of hepatic MTP deficiency [80]. A recombinant adenovirus was used to overexpress MTP in wild-type mice, resulting in increased hepatic VLDL production [81]. Abetalipoproteinemia is an attractive candidate for the development of gene therapy, because even a relatively low level of MTP expression in the liver would be expected to result in some VLDL production and therefore a substantial improvement in vitamin E transport and potentially slowing or halting the progression of neurological and retinal symtoms.

Issues relevant to the development of gene therapy for lipid disorders
Effective gene therapy for the lipid disorders described above will require stable gene expression. Recent progress in vector development has focused on the development of vectors with a potential for long-term expres-

sion. Recombinant adenoviral vectors elicit a cellular immune response to adenoviral proteins, limiting gene expression and posing safety issues. Helper-dependent adenoviruses are fully deleted of adenoviral genes, thus removing the trigger of the immune response. A single intravenous injection of helper-dependent adenovirus results in less hepatotoxicity and considerably longer hepatic expression than earlier-generation adenoviral vectors [82]. Neutralizing antibodies against adenoviral capsid proteins are also generated by the injection of recombinant adenoviruses, preventing successful re-administration [83]. Transient immunosuppression at the time of vector administration permits subsequent administration and repeat transgene expression in liver [84]. The use of different adenoviral serotypes might also provide a strategy for overcoming neutralizing antibodies [85].

Recombinant AAV is an attractive vector for gene transfer because, like the helper-dependent adenovirus, it does not contain sequences encoding viral proteins [86]. Gene expression in the liver by using AAV vectors is dependent on the promoter [87]. AAV was used to express human factor IX in liver at therapeutic concentrations providing the stable correction of hemophilia B in mice and dogs [88,89]. AAV was also used to express human factor IX in muscle to achieve therapeutic plasma concentrations in mice, dogs, and humans [28]. Although AAV is promising, higher levels of gene expression will probably be required for the therapy of most of the lipid disorders described above.

Conclusion

Lipid disorders are common and associated with serious consequences such as atherosclerotic vascular disease and others specific to individual disorders. Current approaches to the treatment of lipid disorders are effective in many but not all patients. New approaches to refractory hypercholesterolemia, severe hypertriglyceridemia, low levels of HDL cholesterol, and certain inherited disorders of intracellular lipid metabolism are needed. Somatic gene therapy is a viable approach to the therapy of several lipid disorders for which therapies are currently inadequate. The molecular etiology and pathophysiology of most of the candidate diseases are very well understood. Animal models exist for the diseases and in many cases preclinical proof-of-principle studies have already been performed. In contrast with many other situations, overexpression of the therapeutic gene is unlikely in most cases to have any deleterious consequences and thus regulated expression is not required. Finally, many of the same genes that could be used to treat genetic deficiency disorders are also attractive candidates for genetic overexpression in persons who do not have a specific gene deficiency disorder but who have other causes of severe hyperlipidemia or low-HDL cholesterol. There has been progress in the development of vectors with less immunogenicity that provide long-term gene expression. The next decade is therefore likely to witness several clinical trials of gene therapy for lipid disorders.

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