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Novel method for reducing plasma cholesterol: a ligand replacement therapy

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Abstract

Despite wide use of statins, significant cardiovascular disease risk persists. High-density lipoprotein based therapy has not yielded any positive results in combating this disease. Newer methods to rapidly decrease plasma cholesterol are much needed. While apolipoprotein B is a ligand for low-density lipoprotein receptor, which clears low-density lipoprotein cholesterol in a highly regulated pathway, apolipoprotein E (apoE) is a ligand for clearing other apolipoprotein B containing atherogenic lipoproteins via an alternate receptor pathway, especially the heparin sulfate proteoglycans on the liver cell surface. We describe here a novel method that replaces apoE as a ligand to clear all of the atherogenic lipoproteins via the heparin sulfate proteoglycans pathway. This ligand replacement apoE mimetic peptide therapy, having been designated as an orphan drug by the US FDA, is in clinical trials.

Keywords

alternate pathway for cholesterol clearance; apoB; apoB-containing lipoproteins; apoE mimetic; CVD; HDL; HMG-CoA; HSPG; LDL-cholesterol; LDL-receptor; ligand replacement therapy; monoclonal antibodies; PCSK-9; SREBP; VLDL

Background

In the year 1856, the German pathologist Rudolf Virchow observed accumulation of lipids in arterial walls [1]. Almost 50 years later, a Russian scientist Nikolai Anitschkow provided experimental support for the lipid hypothesis by feeding rabbits a high-cholesterol diet to develop lesions in the arteries [2]. The lipid hypothesis was still not readily accepted since similar experiments did not work in dogs and rats. The cholesterol hypothesis was accepted in 1976 as the root cause for atherosclerotic lesion formation [3], although the mechanism was not known. Since mortality due to cardiovascular disease was considered to be the

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major cause death in the USA, the lipid hypothesis received significant attention. The NIH sponsored Lipid Research Clinics Coronary Primary Prevention Trial examined the effect of cholesterol reduction in a large study that demonstrated a 2% reduction in the risk of cardiovascular disease (CVD) for each 1% reduction in total plasma cholesterol [4]. This study resulted in the conclusion that it was time to treat cholesterol seriously. In 1985, a National Cholesterol Education Panel (NCEP) was established [5].

During the same time, a milestone in the fields of cell biology and lipid metabolism was achieved by Drs. Michael Brown and Joseph Goldstein who discovered low-density lipoprotein (LDL) receptor pathway and its role in cholesterol homeostasis [6]. This work was recognized by the award of the Nobel Prize in Physiology and Medicine in 1985. Cholesterol metabolism via the LDL receptor mediated pathway is highly regulated [7]. LDL receptor expression on cell surface is regulated by a feedback mechanism based on the level of cellular cholesterol. As cellular cholesterol is increased, LDL receptor expression is decreased. The subsequent discovery of sterol regulatory element binding proteins (SREBPs) and their functions clarified how cellular cholesterol is involved in the regulation of LDL receptor levels [8].

The enzyme HMG-CoA reductase catalyzes a rate-limiting step in cholesterol biosynthesis. The discovery of small molecule inhibitors of HMG-CoA reductase that block the synthesis of cholesterol by Akira Endo is another milestone in reducing the risk for atherosclerosis [9]. HMG-CoA reductase inhibitors, which became known as statins, increase the expression of the LDL receptor by blocking cellular synthesis of cholesterol. This in turn takes up more LDL cholesterol from the circulation, and thus reduces the amount of cholesterol circulating in the blood, and the cholesterol rich lipoproteins retained in the artery wall [10]. A meta-analysis of 14 randomized statin clinical trials conducted between 1994 and 2004 demonstrated that the cholesterol reduction through the use of statins has only reduced the incidence of CVD by 30% [11]. This prompted suggestions that earlier and more aggressive cholesterol lowering therapy may provide added benefit [12]. However, a more recent meta-analysis of randomized statin clinical trials indicates that more aggressive cholesterol lowering still only reduced risk by 40–50% [13]. The high residual incidence of CVD stimulated continued research to discover newer methods of preventing and treating CVD; by, among others, directly reducing inflammation in the artery wall, decreasing retention of cholesterol, increasing HDL levels and/or decreasing circulating cholesterol levels by further enhancement of the reverse cholesterol transport process in which cellular cholesterol enters the plasma compartment for clearance by the liver [14–16].

Significant effort on HDL therapy has shown that the HDL story is very complicated and much more research is needed to understand what HDL is and its main anti-atherogenic function. This complexity is reflected in the failure to achieve decreasing in CVD in several clinical trials aimed at increasing HDL levels [17]. In addition, two large studies examining genetic risk factors for cardiovascular disease have indicated that HDL cholesterol is not an independent risk factor for CVD [18,19]. Moreover, biomarkers for determining if HDL therapy is working in patients, requires additional research. Thus in most of the CVD patients, reduction of plasma cholesterol, which can be easily followed, remains the only

cholesterol modifying intervention that has been shown to be beneficial. New drugs aimed at reducing plasma cholesterol are much needed.

Preventing the synthesis of VLDL by the liver is an LDL receptor independent approach to reducing plasma cholesterol [16]. Inhibition of the synthesis of apoB or of microsomal triglyceride transfer protein (MTP) results in significant reductions in plasma cholesterol [20,21]. Unfortunately, prevention of VLDL synthesis and secretion by the liver has the side effect of increasing hepatic triglyceride content [22,23]. However, the importance of an LDL receptor-independent mechanism for cholesterol reduction in patients with homozygous familial hypercholesterolemia has resulted in the regulatory approval to two new VLDL synthesis inhibiting drugs to provide additional treatment options for these high-risk patients.

Recently it has been shown that subjects who possess high amounts proprotein convertase subtilisin-kexin type 9 (PCSK-9), have high levels of circulating plasma cholesterol [24]. This is due to the ability of PCSK-9 that is secreted into plasma by hepatocytes to degrade LDL receptors, inhibiting recycling of receptors to the cell surface. This process thus inhibits uptake of plasma LDL [25]. People with a loss of function defect in the *PCSK-9* gene possess less cholesterol since this mutated protein does not degrade LDL-receptors. As a result, PCSK-9 has become an important cholesterol reduction target [26], and two current strategies for lowering cholesterol by decreasing the amount of circulating PCSK-9 are currently in clinical development: 1) inhibition of PCSK-9 synthesis with antisense nucleotides (ASOs) and RNA interference (siRNAs) or 2) inhibition of the binding of PCSK-9 to the LDL-receptor via administration of monoclonal antibodies (mABs) specific for PCSK-9 [27,28].

Two mABS specific for PCSK9 are now in advanced clinical development. Alirocumab (Regeneron/Sanofi) has demonstrated LDL cholesterol reductions of greater than 60% in patients on stable statin therapy, and almost 60% in patients with heterozygous familial hypercholesterolemia when dosed at 150 mg every 2 weeks [29]. AMG145, the Amgen/Pfizer antibody, was similarly efficacious when dosed at 140 mg every 2 weeks, but reduced LDL cholesterol by 50% when dosed at 420 mg monthly. ALN-PCS, the Alnylam siRNA that targets PCSK9 synthesis, is much earlier in the development cycle. A dose of 0.4 mg/kg decreased PCSK9 activity by 70% and lowered LDL cholesterol by 40% in a Phase I study in healthy volunteers. It should be noted that the PCSK9 antibodies are less effective lowering VLDL and triglycerides than lowering LDL. This is not unexpected since VLDL remnants are cleared via apoE [30].

ApoE as a target for cardiovascular disease

Among the two apolipoproteins that are anti-inflammatory, administrations of apoA-I, and recombinant HDL does not change the plasma cholesterol levels while, as will be discussed later, apoE possesses both anti-inflammatory and plasma cholesterol reducing properties. A recent review describes in detail the anti-atherogenic and anti-inflammatory properties apoA-I and apoA-I mimetic peptides [31]. This article will focus on apoE as a ligand for

alternative receptors, the peptide mimetics based on the properties of apoE, and we will describe how apoE mimetics supplement or replace apoE as a ligand.

ApoE provides an alternative approach to enhancing the clearance of cholesterol rich lipoproteins. While apoB-100 on LDL possesses a receptor binding domain that is specific for LDL receptor, apoE is an alternative ligand for clearing atherogenic lipoproteins through a different receptor pathway. Indeed, it has been shown that lipoproteins containing apoE bypass the LDL receptor and clear these lipoproteins via either LDL-receptor related proteins (LDL-R) or highly abundant cell surface proteoglycans (HSPG) [32]. HSPG on the cell surface, with abundant carboxyl and sulfate groups, represents a unique and highly versatile receptor for the endocytosis of macromolecules and is well described previously [33]. Mahley and Ji [30] state that apoE is critically important to sequestration of VLDL remnant lipoproteins in the space of Disse, which is rich in the heparan-sulfate proteoglycan receptor (HSPG). MacArthur *et al.* have convincingly shown that HSPG pathway for clearance of lipoproteins is independent of LDL receptor pathway but also have shown that it may play a role in LDL clearance. [34]. Stanford *et al.* and Chen and Williams have demonstrated that Syndecan-1 is the HSPG responsible for apoE-mediated remnant lipoprotein clearance in mice [35,36]. Mahley has also described apoE as a cholesterol transport protein that also exhibits an expanding role in cell biology [37]. It has been shown that infusion of apoE in cholesterol fed rabbits caused accelerated clearance of remnant lipoproteins [37]. ApoE null mice possess defective clearance of atherogenic lipoproteins and develop atherosclerosis even on a normal diet. Bone marrow from wild type mice was injected into irradiated apoE^{-/-} mice. After 4 weeks, serum apoE levels reached 12.5% of levels measured in normal C57BL/6 mice [38]. This level of apoE was associated with a 74% reduction in plasma cholesterol compared with baseline cholesterol measurement prior to transplantation [39]. Further, aortic lesions were significantly reduced in these mice 3 months after initiating feeding with a Western diet.

Human LDL-possesses only one apolipoprotein, apoB-100, which is integral to the structure of LDL. The receptor binding domain. RLKRGLK (apoB [359–367]) is highly positively charged. Since apoB is a very large protein (4536 amino acids), each LDL particle would possess one LDL receptor binding domain. Furthermore, as mentioned earlier, LDL receptor mediated pathway is highly regulated. In contrast, apoE is a smaller protein with 299 amino acids, and readily transfers between cholesterol rich lipoproteins. The receptor binding domain of apoE also possesses highly positively charged amino acid residues with the sequence LRKLRKRLLR (141–150 of apoE protein). However, as discussed earlier, apoE-containing lipoproteins bypass the LDL receptors and clear atherogenic lipoproteins via the HSPG pathway that is not so highly regulated by cellular cholesterol content.

Design of peptide mimics of apoE

ApoE possesses a dual-domain nature, an N-terminal receptor binding domain and a lipid binding domain consisting of a long amphipathic helical domain (203–266 region of apoE) at the C-terminus of apoE. It was hypothesized that apoE binds to VLDL and remnant lipoproteins with a larger curvature because of this long amphipathic helical domain [40]. We hypothesized that if the receptor binding domain consisting of 10 amino acids is

attached to a shorter amphipathic helical domain, the resulting short peptide would associate with all of the atherogenic lipoproteins, including LDL, and clear them rapidly via the HSPG pathway [41].

Our experience with extensive studies of apoA-I mimetic peptides over two decades allowed us to select an 18 residue peptide (18A) designed in our laboratory [42] as the lipid-associating domain. We then covalently linked LRKLRKRLLR to 18A and protected the ends to obtain Ac-hE18A-NH₂, wherein hE stands for LRKLRKRLLR. Since the receptor-binding domain in other species was similar but with a change in only one amino acid, several other peptides with similar sequence and a sequence in which Lys in receptor binding domain was replaced by Arg (LRRLRRRLLR) were synthesized. Cell culture studies demonstrated that all of these peptides enhanced binding of LDL and VLDL (isolated from human plasma) several fold. In addition, degradation of heparan sulfate abolished peptide-mediated binding and clearance, thus supporting our hypothesis that the peptides derived to mimic apo E function enhanced clearance *via* the HSPG pathway [43]. Additional support for this conclusion came from the binding studies that showed that peptide-mediated binding of LDL and VLDL to fibroblasts and hepatocytes was not saturable. In contrast, in the absence of these peptides, binding of LDL to these cells has been shown to be saturable due a single binding domain for the LDL receptor and to the presence of limited number of LDL receptors on the cell surface.

AEM-28 is highly efficacious in reducing plasma cholesterol, reducing inflammation & inhibiting atherosclerosis

Among the first set of peptides, Ac-hE18A-NH₂ (also known as AEM-28) has been most extensively studied. Further support for the HSPG mediated uptake mechanism came from studies in Watanabe heritable hyperlipidemic (WHHL) rabbits that possess a genetic defect in the LDL receptor [44]. These rabbits do not clear LDL, and develop hypercholesterolemia and atherosclerotic lesions due to a functional defect in LDL receptor that is similar to that seen in some humans with homozygous familial hypercholesterolemia. A single administration of AEM-28 cleared plasma cholesterol by more than 50% and cholesterol remained lower than normal levels for more than 18 h. Plasma lipoprotein profiles indicated rapid clearance of both LDL and VLDL-like lipoproteins. In addition, administration of the peptide decreased levels of lipid hydroperoxide and increased the plasma paraoxonase-1 (PON-1) activity. These changes correlated well with the decrease in plasma cholesterol. Endothelial function was also significantly improved compared with untreated WHHL rabbits, perhaps due to both the cholesterol reduction and noncholesterol reducing anti-atherogenic properties of this peptide, analogous to apoE [36]. Since the plasma clearance rate is very fast [45], the peptide was not found to be immunogenic.

Several reports suggest that anti-inflammatory effects of the apoE are independent of its cholesterol-lowering property [39–40,46–48]. ApoE can interact with and neutralize endotoxin, resulting in a reduction in plasma cytokine levels and mortality due to sepsis [49]. It is therefore possible that polymorphisms in the apoE gene may result in enhancement of the inflammatory response and an increase in mortality in animal models and patients with sepsis [50,51]. ApoE null mice, compared with normal mice, have

significantly elevated levels of pro-inflammatory cytokines and correlate with increased mortality in mice treated with either LPS or infected with bacterial pathogens [41–52]. Our observation of increased PON-1 activity and improvement in endothelial function support the concept that AEM-28 also possesses anti-inflammatory properties that are independent of its plasma cholesterol reducing properties [36].

Raffai *et al.* [53] used a hypomorphic apoE mouse to study the effects of low levels of apoE on atherosclerotic lesion formation. The low levels of apoE expressed in these mice significantly decreased the lipid rich layer of foam cells in the aorta, independent of plasma cholesterol. Further studies by Raffai and colleagues indicate that apoE expression decreases circulating leukocytes and pro-inflammatory Ly6c^{high} monocytes, decreasing activation of monocytes by neutral lipids, decreased surface expression of adhesion molecules and decreased the expression of inflammatory molecules on endothelial cells, all independent of plasma cholesterol [54].

In support of this, we have shown that multiple administrations (three-times a week for 8 weeks of 100 µg/mouse) of a single domain cationic peptide mR18L is also capable of reducing plasma cholesterol to a similar extent to that of AEM-28 at the end of the experiment in LDL-R null mice on Western diet. However, AEM-28 was much more effective in reducing aortic lesions and macrophage load compared with mR18L despite the cholesterol levels being similar [55]. Furthermore, the levels of lipid hydroperoxides were lowered in the AEM-28 group and not in mR18L group.

Decreasing inflammation, macrophage activation and macrophage load in the artery wall, independent of plasma cholesterol, should result in protection from atherosclerosis. This prompted us to examine if the peptide exhibits a sustained effect even after the withdrawal of the drug. Due to the recycling nature of AEM-28 in our studies, we hypothesized that AEM-28 would exhibit a prolonged beneficial effect on the endothelium [56]. To test this hypothesis, apoE null mice were fed a Western type diet for 6 weeks to enhance the rate of lesion formation. During the Western type diet feeding, their cholesterol levels were raised to 1400 mg/dl. Changing this to normal diet for 2 weeks brought cholesterol levels back to that which are normal for apoE null mice on a chow diet. AEM-28 was then administered for 4 weeks and then withdrawn for 4 weeks before analysis of aortic lesions. Compared with the control group of animals, the peptide-administered group exhibited significantly less atherosclerotic lesion [57]. The decreased lesion expression was independent of plasma cholesterol levels at the end of peptide treatment.

These studies provide strong support for the use of this peptide in patients who are resistant to statins and in refractive hyperlipidemic patients. AEM-28 has been granted orphan drug designation for the treatment of homozygous familial hypercholesterolemia by the FDA. Clinical development of AEM-28 began in the first quarter of 2014. The in-life component of a single ascending dose Phase 1a and multiple ascending dose Phase 1b clinical study was completed at the end of 2014.

Conclusion

Despite impressive reductions in plasma cholesterol with statins and statins in combination with other cholesterol lowering drugs, CVD remains a major cause of morbidity and mortality in Western nations. New cholesterol lowering therapeutics have the potential to further lower plasma cholesterol levels and to provide additional treatment options for high risk patient populations. Nonetheless, therapeutic modalities that alter plasma cholesterol levels and also directly decrease artery wall inflammation and atherosclerosis are needed. Considering the recent failures of apoA-I and HDL-targeted therapies and the central role that apoE plays in lipid metabolism, time is prime for targeting apoE for future drug development for this devastating disease. Since 28 residue peptide AEM-28 mimics most of the properties of 299-residue full length protein, readily inserts onto the surface of all apoB containing lipoproteins, and can be produced in large amounts, and the peptide AEM-28 has shown efficacy in several animal models and Phase 1 clinical trials, apoE mimetic peptides hold promise as a future therapy for CVD.

Future perspective

The centrality of apoE in resolving inflammation due to various lipid-mediated disorders is clearly gaining importance. Recently it was reported that PCSK9 inhibition fails to alter hepatic LDLR, circulating cholesterol, and atherosclerosis in the absence of apoE. [58]. While PCSK9 is able to reduce LDL cholesterol, apoE mimetics clear all of the atherogenic lipoproteins (Figure 1). With advancement in the ligand replacement therapy, it is possible that newer apoE mimetic peptides that are orally administrable can be synthesized. The results on sustained effect may indicate that patients can be intermittently treated to reduce atheroma burden. It is anticipated that the dosage can be adjusted depending on the levels of plasma cholesterol. Since reduction in plasma cholesterol also reduces several inflammatory diseases such as Alzheimer's disease and diabetes, the apoE mimetics are expected to be used as therapy for several lipid-mediated disorders.

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Executive summary

- Despite decades of cholesterol lowering with multiple drugs, significant risk for cardiovascular disease persists.
- LDL receptor mediated pathway is highly regulated. Recent research on PCSK-9 inhibitors has improved the clearance ability of LDL cholesterol significantly.
- However, clearance of chylomicrons, remnant particles and VLDL still require newer and improved strategies.
- While apoB is the ligand for LDL receptor, presence of apoE on lipoprotein particles clears all of the atherogenic lipoproteins more efficiently, bypassing the LDL receptor pathway.
- ApoE acts as a ligand to cell surface heparan sulfate proteoglycans on the liver. Administration of isolated apoE into high cholesterol fed rabbits dramatically clears cholesterol, thus inhibiting the lesion formation.
- However, apoE is a large protein with 299 amino acid residues and is expensive to either isolate or produce via the molecular biology techniques.
- The present article describes a 28 residue peptide, called AEM-28, that can be easily be synthesized in large scale and has been shown to mimic the anti-atherogenic and cholesterol reducing properties of apoE.
- This peptide thus replaces apoE as a ligand for the clearance of all of the apoB-containing atherogenic lipoproteins.
- This peptide drug is undergoing clinical evaluation in humans.
- Since reduction in plasma cholesterol also reduces several inflammatory diseases such Alzheimer's disease and diabetes, the apoE mimetics are expected to be used as therapy for several lipid-mediated disorders.

Figure 1. AEM-28 binds to all of the atherogenic lipoproteins to enhance the uptake of these lipoproteins by the liver

Shown diagrammatically are the various receptors for the uptake of lipoproteins present in the liver. LDL uptake is highly specific. ApoE acts as a ligand to alternative receptors on the liver surface. ApoE mimetic is able to replace both apoE and apoB as a ligand to enhance the binding and uptake of atherogenic lipoproteins via the HSPG receptors.

AEM-28: Ac-hE18A-NH₂; ApoE: Apolipoprotein E; IDL: Intermediate density lipoprotein; SRB1: Scavenger receptor type B1.