HDL cholesterol: atherosclerosis and beyond
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Apolipoprotein A-I mimetic Peptides

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Abstract

Objective
To review published data related to the potential applicability of apolipoprotein AI mimetic peptides.

Recent findings
Despite a wealth of information on HDL-c levels and risk for CVD, little evidence is present to suggest that raising HDL-c levels per se will result in CVD risk reduction. Rather, increasing HDL functionality might be a more successful strategy to reverse the process of atherosclerosis. In as such, apoAI mimetic peptides, either in single or tandem formulation, hold great promise. Evidence gathered over the last years has provided insight in the extent to which mimetics influence several cardio metabolic pathways. ApoA-I mimetics have shown to have anti-inflammatory, antioxidant, and antiatherogenic effects. Direct comparisons between different mimetics have provided insight in factors influencing the differential beneficial consequences of these peptides.

Data derived from recent studies suggest that mimetics might also gain their position as a therapeutic intervention in the treatment of septicaemia, transplantation rejection, diabetes and other auto-immune diseases.

Summary
This review provides a summary of the current literature on the potential application of apoAI mimetics as therapeutic agents. There is increasing evidence that these mimetics should be considered as a promising supplement to current strategies. Results from human studies addressing the in vivo effects of the different apoAI mimetics are eagerly awaited.
Introduction

Since one of the first reports in 1951,1 a plethora of prospective epidemiological studies have shown that plasma HDL-c levels and the risk for cardiovascular disease (CVD) are inversely related.2 The magnitude of the association is quantified in a widely cited meta-analysis showing that a 1 mg/dl increase of HDL-c level is associated with a 2–3% decrease in CVD risk.3

A number of mechanisms by which HDL-c may influence the process of atherosclerosis has been described. (reviewed in Kontush et al 20064). Fielding and Fielding5 demonstrated in 1982 that HDL can act as an acceptor of cellular cholesterol, which is proposed to constitute the first step in a hypothetical pathway that is known as reverse cholesterol transport (RCT). RCT is defined as the uptake of cholesterol from peripheral cells by lipid-poor apoA-I and HDL, and the subsequent delivery to the liver for excretion into the feces. The function of HDL as a atheroprotective particle extends well beyond this role in cholesterol transport; it has also been found to have anti-apoptotic,6 antiinflammatory7 and antithrombotic8 capacities.

Cardiovascular disease remains a major cause of morbidity and mortality, despite interventions to decrease the number or severity of known risk factors. As a consequence, HDL-c increasing therapy is considered a suitable target for CVD prevention. Indeed, infusion of exogenous HDL has been shown to result in improvements in hallmarks of atherosclerosis, such as endothelial function,9 coronary atheroma volume10 and plaque morphology.11 These studies, however, were small scaled and should be regarded as a proof of concept that an increase in HDL-c does change biomarkers of atherosclerosis. Repetitive infusions of HDL are not considered a feasible therapy for the wide scaled burden of CVD; it would require large resources to produce considerable quantities of this lipoprotein. Consequently, large efforts have been put in the strive for orally administered HDL-c increasing medication. Several of these are available (i.e. fibrates or nicotinic acid derivates). Most of these drugs, however, do not exclusively raise HDL, but have beneficial effects on other risk factors as well, and as such, the contribution of the HDL-c increase per se has been impossible to delineate.

However, efforts in the field of HDL therapeutics have resulted in the recent development of Cholesteryl ester transfer protein (CETP) inhibitors. This class of drugs induces significant HDL-c increases. The ILLUMINATE trial, in which 15.000 individuals were randomized to either statin or statin combined with the CETP inhibitor torcetrapib, did show increased mortality amongst the torcetrapib treated patients, despite an impressive (>100%) increase in HDL-c.12-14 The result is counterintuitive, and the underlying mechanism has not been fully elucidated, but off target effects of the torcetrapib molecule are the most likely explanation to date.

After Illuminate, a subtle shift in the HDL-atherosclerosis paradigm has occurred. Whereas in the past the main focus was to increase HDL-c levels per se, the concept has changed to increase HDL functionality.15,16

With this new paradigm kept in mind, apolipoproteinA-I (apoA-I) should be considered an attractive target. ApoA-I is the structural protein of the HDL particle and it plays a crucial role in many of the beneficial effects of HDL. Studies in rodents have established that apoA-I does play a role in atherosclerosis, although the magnitude of the effect differed amongst the studies, which might be in part attributable to the genetic background of the animals and additional...
The development of ApoA-I mimetic peptides

Apolipoprotein AI is a relatively large protein, comprising of 243 amino acids in 10 amphipathic alpha helices, of which most are separated by a proline residue. Due to its size, apoAI would require parenteral administration. As a consequence, it was compulsory to design smaller mimetic peptides, without losing the lipid binding and antiatherogenic capacities of these molecules. In 1985, Anantharamaiah and coworkers produced an 18 aminoacid containing peptide folded into an alpha-helix. The peptide did not have direct sequence homology to either one of the helices of the apoAI protein, but the secondary structure of apoAI was replicated. This so called 18A peptide was found to have similar properties to the apoA-I helices in terms of charge distribution and lipid-binding capacity. Cellular efflux-inducing capacity of 18A was shown to be similar to apoA-I in an assay where cultured mouse fibroblasts were used. In addition, like apoA-I, 18A was shown to activate Lecithin:Cholesterol Acyltransferase (LCAT), a pivotal enzyme in HDL maturation. As such, 18A was thus shown to have quite similar properties in lipid metabolism as apoA-I.

In an attempt to optimize the peptide, stability was improved by replacing the existing nonpolar amino acids on 18A with phenylalanine (F) residues. The increasing number of F residues (resulting in 2F, 3F, 4F, 5F, 6F and 7F, reflecting the number of F residues) did result in increased lipid affinity and hydrophobicity; 6F and 7F were the most hydrophobic, but these peptides were shown to lose the affinity to bind phospholipids, and in subsequent experiments the main focus has been on the 4F and 5F peptides.

Peptides can either be synthesized from L-aminoacids (i.e. L-4F) or D-aminoacids (D-4F). In vitro properties have been shown to be similar for both isomers, but the L isomer is significantly more prone to proteolysis compared to the D isomer, since mammalian enzymes recognize peptides made from L-aminoacids, but not from D-aminoacids.

In addition to lipid binding and hydrophobicity, peptides have been tested for their antiinflammatory and antioxidant characteristics. A commonly described method is the one...
described by Navab and coworkers, who have used a model in which LDL-c induced activation of monocyte chemotactic activity was tested in an in vitro arterial wall coculture model. In these models mimetic peptides are compared with each other and with apoA-I. Attempts to mimick apoAI in all its anti-atherogenic effects have thusfar not been successful, but mimetics have been shown to be superior to apoA-I when it comes to specific characteristics. 4F, for example, was shown to bind oxidized lipids with orders of magnitude higher affinity compared to apoA-I. This finding might explain why these peptides have biological effects despite low plasma concentrations. The variation in effects between peptides and apoA-I also suggests that “mimetics” might not be a proper nomenclature.

**Tandem helical mimetic peptides**

As said above, apoA-I contains multiple helices linked by a proline residue. Single helix peptides have been shown to be efficient antiatherogeneic molecules, but tandem helical mimetic peptides have been created in an attempt to further increase efficacy by more closely mimicking the apoA-I peptide.

In a direct comparison, Wool and coworkers showed that symmetrical peptides, composed of two 4F peptides linked by a proline or alanine residue, were more efficient in a cholesterol efflux from lipid-loaded murine macrophages compared to the single 4F peptide. Copper-mediated oxidation of purified mouse LDL was inhibited by the 4F peptide, but the tandem peptides increased oxidation, clearly emphasizing the variability of effects between the single and tandem peptides in in vitro assays.

Recently, 22 bihelical apoA-I mimetic peptides were investigated in vitro for their capacity and specificity of cholesterol efflux, and their inhibiting effects on inflammation and LDL-oxidation. In this comprehensive first systematic analysis of multiple structural modifications none of the peptides tested were found to be equally effective in all antiatherogenic functions.

Moreover; the anti-inflammatory, antioxidant and efflux capacities were found to be related to differential structural features of the peptides. For efflux efficiency and specificity, for example, mean hydrophobicity, charge, size and angle of the link between two helices were crucial, whereas for antioxidant properties the presence of cysteine and histidine residues was important. The latter has also been proposed as a potential explanation for the increased antioxidant property of apoA-I Milano, a genetic variant known to be associated with reduced risk for CVD.

The fact that none of the peptides tested could outcompete the beneficial functions of apoAI in all facets of antiatherogeneity suggests that different portions of the apoAI peptide might be involved. As a consequence, mimicking one part of apoAI does not necessarily result in an overall beneficial property of the designed apoAI mimetic peptide. A combination of different mimetic peptides, harboring various beneficial effects, might therefore be a prerequisite to mimick the full spectre of antiatherogenic characteristics of apoAI.

One could also envision that tailored therapy can be achieved by the combination of different peptides.
ApoA-I mimetics in animal studies

The initial \textit{in vivo} studies addressing the effect of apoA-I mimetics were performed by Garber and coworkers. In this study, C57Bl/6J mice were put on an atherogenic diet and 5F was administered daily by intraperitoneal injection at a dose of 20 microg/day for 16 weeks. Mice treated with PBS or murine apoAI (50microgram/day) were used as controls. Lipids and lipoproteins were not significantly altered upon 5F injections and administration of 5F was found to be non toxic. Importantly, no antibodies to the injected materials were observed. The aortic atherosclerotic lesion area was significantly less (44% reduction) in 5F treated mice compared to mice receiving placebo or murine apoA-I. HDL isolated from 5F injected mice was shown to be superior to HDL derived from PBS and apoAI treated mice in terms of reduction of monocyte chemotaxis and lipid hydroxyperoxidase formation. This, in combination with the finding that lipid levels were not changed by 5F injection, shows that functionality of the HDL pool was enhanced by 5F.

In subsequent \textit{in vivo} studies, the main focus has been on 4F. This is in part due to the fact that D-4F can be administered orally in drinking water or by gavage. In apoE knockout mice as well as in LDL-r KO mice, D-4F was shown to decrease the aortic root lesions by more than 70%, despite very low bioavailability and low plasma concentrations. The marked reduction in atherosclerotic lesions occurred independent of changes in total plasma or HDL-cholesterol. However, administration of D-4F in the drinking water resulted in a substantial change of HDL from a pro- to an anti-inflammatory particle, emphasizing that D-4F therapy induced a qualitative rather than a quantitative effect on HDL. In a subsequent study, this suggestion was confirmed by showing that administration of D-4F caused a change in HDL distribution towards pre-beta HDL (a fraction known for its efflux efficacy), and that it increased HDL associated paraoxonase activity.

Low doses of D-4F and pravastatin work synergistically on HDL-c level, HDL function and atherosclerosis prevention in an apoE null mice model. This might be relevant for future clinical perspectives.

Bielicki and co workers published the beneficial effect of a new HDL mimetic single helix peptide (ATI-5261) in LDL-r KO mice and apoE null mice on a 13-18 week during high-fat Western diet. Daily intraperitoneal injections of ATI-5261 (30 mg/kg) for 6 weeks reduced atherosclerosis, as judged by lesion area covering the aorta, by 30% and 45% in LDL-R -/- and apoE -/- mice respectively. Interestingly, one single intraperitoneal injection of ATI-5261 was found to increase reverse cholesterol transport from macrophage foam-cells to feces over 24-48 h.

Emerging perspectives for apoA-I mimetics

The effects of apoA-I mimetics is not restricted to dyslipidemic mice models. In a recent study, Vaziri and coworkers showed the benefit of L4F (5 mg/kg s.c. 3 times weekly for 4 weeks) on inflammation and oxidative stress in a chronic kidney disease (CKD) rat model. L4F attenuated a large number of the markers for inflammation and oxidation (such as NAD(P)H oxidase subunits, COX-2, 12-lipoxygenase, MCP-1, PAI-1, myeloperoxidase)
in the thoracic aorta without altering plasma lipids. Compared to WT mice, ApoE-deficient mice have been shown to suffer significantly more from signs of renal dysfunction such as proteinuria, tubulo-interstitial inflammation, and mesangial expansion. Oral D-4F administration was recently found to have favorable effects on all of these characteristics. Further studies in CKD patients are awaited to address whether apoAI mimetics hold promise for atherosclerosis regression and preservation of kidney function.

Another patient population that might benefit from apoA-I mimetics are septic patients. Apolipoproteins and HDL have been shown to beneficially change the inflammatory response to lipopolysaccharide (LPS), and a recent study showed that intraperitoneal injection of 4F significantly blunted the hypotensive response to LPS (LPS treated rats: 34% decrease, LPS and 4F treated rats: 17% decrease in systolic blood pressure). In order to unravel the underlying mechanism, aortic ring segments from LPS-treated rats were isolated. These ex vivo studies showed a reduced contractile response to phenylephrine in aortae of LPS-treated rats compared to controls, and this reduced contractility was reverted by 4F via nitric oxide synthase 2 (NOS2) downregulation. The concentration of circulating endotoxin was significantly reduced in 4F treated rats, and interestingly, HDL-c levels increased in the LPS+4F treated animals (38 to 45mg/dl). Similar to other studies, an HDL-c reduction was found in LPS+vehicle treated rats (38 to 28mg/dL).

The findings of this study are in line with previous studies showing that the apoA-I mimetic 4F significantly inhibits the induction of pro-inflammatory mediators by LPS in cultured endothelial cells. Interestingly, 4F was shown to reduce 24hour mortality in LPS- treated rats (60% mortality in LPS and 10% in LPS+4F, total 40 animals).

In addition, transplant-associated vasculopathy is reverted upon intraperitoneal D-4F administration in a mouse heart transplant model. The authors hypothesize that this is partly mediated through D-4F induced HemeOxygenase-1 upregulation.

In a mouse lupus model, L-4F was not only shown to reduce atherosclerosis progression, but it also resulted in a reduction of IgG anti-dsDNA, proteinuria and glomerulonephritis, suggestive of protective effect on lupus-like disease. Beneficial vascular effects have also been described in a systemic sclerosis model.

Administration of L-4F (2mg/kg/d) to ob/ob mice reduced adiposity, improved insulin sensitivity, and improved glucose tolerance, which might be related to apoAI mimic induced increase in uncoupling protein 1 (UCP1) mRNA and protein levels as well as the stimulation of AMPK phosphorylation in brown adipocytes in culture.

Other favorable effects of mimetics have shown to be a reduction of platelet aggregation, an increase of cognitive function in an Alzheimer’s mice model and prevention of fibrosis after onset of steatohepatitis.

**ApoA-I in human studies**

Based on all intriguing beneficial findings in animal studies, apoA-I mimetics hold promise for human therapy. Apart from its anticipated efficacy, apoA-I mimetics have more positive characteristics: they are safe, well tolerated and relatively inexpensive compared.
Moreover, the size of the peptide does implicate that administration might be possible in an oral formulation, which is a prerequisite for a long term treatment in large numbers of patients and modifications for optimal oral delivery are in development.

The first clinical trial of oral D-4F was performed in CAD and high risk patients who received a single dose of 30, 100, 300, or 500 mg of D-4F (n = 8 for each dose) or placebo (n = 8) under fasting conditions. Ten additional patients received 500 mg (n = 8) or placebo (n = 2) with a low-fat meal. The Tmax was shown to be 30 minutes and D-4F was detectable in plasma at all dosages. The single dose was well tolerated and shown to be safe. No effect on lipids and lipoproteins was found, but the anti-inflammatory index, assessed by comparing the ability to inhibit LDL-induced monocyte chemotactic activity in cultures of human aortic endothelial cells, increased in the highest doses compared to placebo. Additional studies focusing on these vascular effects are expected.

**Other apoA-I based therapies.**

Apart from apoA-I mimetics, several other apoA-I based therapies have emerged and these have reached different stages of research. Full-length apoA-I, recombinant HDL, apoAI enhancers and active delipidation are amongst these therapies. Results from studies with these are eagerly awaited.

**Conclusion**

Recent studies have increased our understanding of the effects of apoA-I mimetics. All peptides studied have been shown to induce beneficial effects on oxidation, inflammation and cholesterol efflux and combining different peptides might be a prerequisite to establish the full spectrum of possible beneficial effects.

Whereas the focus for apoA-I mimetics has traditionally been directed towards atherosclerosis, recent studies have shown effects in several other disease states. These provocative findings do require further investigations in carefully designed clinical trials in humans.
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