Published in final edited form as:

J Clin Lipidol. 2007 May ; 1(2): 142–147.

Peptide Mimetics of Apolipoproteins Improve HDL Function

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Abstract

Over the past decade evidence has accumulated that suggests that the anti-inflammatory properties of HDL may be at least as important as the levels of HDL-cholesterol. The recent failure of the torcetrapib clinical trials has highlighted the potential differences between HDL-cholesterol levels and HDL function. Agents to improve HDL function including HDL anti-inflammatory properties provide a new therapeutic strategy for ameliorating atherosclerosis and other chronic inflammatory conditions related to dyslipidemia. Seeking guidance from the structure of the apolipoproteins of the plasma lipoproteins has allowed the creation of a series of polypeptides that have interesting functionality with therapeutic implications. In animal models of atherosclerosis, peptide mimetics of apolipoproteins have been shown to improve the anti-inflammatory properties of HDL, significantly reduce lesions and improve vascular inflammation and function without necessarily altering HDL-cholesterol levels. Some of these are now entering the clinical arena as interventions in pharmacologic and pharmacodynamic studies.

Normal HDL Reduces LDL-induced Monocyte Chemotactic Activity

In 1991 Navab et al reported that adding LDL to co-cultures of human aortic endothelial cells and smooth muscle cells induced the artery wall cells to secrete the potent monocyte chemoattractant MCP-1 that was prevented by the addition of normal HDL (1). Upon incubation of LDL with the artery wall cells it was found that oxidized lipids were generated that were responsible for inducing the artery wall cells to produce MCP-1 and that these oxidized lipids could be removed from LDL by defatted albumin (2). In subsequent studies Navab et al. found that when apolipoprotein A-I was incubated with normal human LDL *in vitro* and then separated from the LDL half to two-thirds of the oxidized lipids associated with the LDL transferred to apoA-I (3). After treatment of the LDL with apoA-I followed by separation from the apoA-I the LDL was no longer able to induce human artery wall cells to produce MCP-1 (3). Injection of human apoA-I into mice that are susceptible to diet-induced atherosclerosis also rendered their LDL unable to induce artery wall cells to produce MCP-1 (3). Similarly, infusion of human apoA-I into human volunteers dramatically reduced the

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ability of their LDL to induce human artery wall cells to produce MCP-1 (3). When human apoA-I was incubated with the artery wall cells and then removed prior to the addition of LDL the LDL was no longer able to induce MCP-1 production (3). However, if the apoA-I was left in the incubation medium in a co-incubation (i.e. not separated from the LDL) the LDL was able to induce MCP-1 (3).

Search for an ApoA-I Mimetic Peptide

Human apoA-I has 243 amino acids. The laboratories of Segrest and Anantharamaiah designed 18 amino acid peptides that did not have sequence homology with apoA-I but possessed the class A amphipathic helical motif present in apoA-I lipid binding domains (4-6). This peptide was called 18A because it contained 18 amino acids that formed a class A amphipathic helix. When the carboxy and amino termini were blocked by addition of an acetyl group and amide group, its stability and lipid binding properties were improved and the peptide was called 2F in recognition of the two phenylalanine residues on the hydrophobic face. While the 2F peptide mimicked many of the lipid binding properties of apoA-I it failed to alter lesions in a mouse model of atherosclerosis (7). Using the human artery wall coculture assay a series of peptides were tested for their ability to inhibit LDL-induced monocyte chemotactic activity. Peptides with the same structure as 2F but with two leucine residues on the hydrophobic face of the peptide replaced with two phenylalanine residues (4F) was found to have superior biologic and physical characteristics (7). Two peptides with biologic activity similar to 4F were found which had 5 and 6 phenylalanine residues on the hydrophobic face of the molecule (5F, 6F) but a peptide with 7 phenylalanine residues on the hydrophobic face (7F) was much less active (7). The peptides 5F and 6F were much less soluble in aqueous solutions than was 4F (8).

ApoA-I Mimetic Peptides in Mouse Models of Atherosclerosis and in Monkeys

Administration of 5F by injection significantly inhibited lesion formation in C57BL/6J mice fed an atherogenic diet without significantly altering lipoprotein profiles (9).

After oral administration of radiolabeled 4F peptide synthesized from L-amino acids to LDL receptor null mice most of the label was found in degradation products (10). However, when the peptide was synthesized from all D-amino acids intact peptide was found in the circulation after oral administration (10). While normal HDL reduced the ability of LDL to induce monocyte chemotactic activity in cultures of human artery wall cells, HDL taken from mouse models of atherosclerosis (apoE null mice or LDL receptor null mice on a Western diet) actually increased the production of monocyte chemotactic activity when added to the cultures with LDL. Oral administration of the peptide synthesized from D-amino acids (D-4F) to LDL receptor null mice on a Western diet rendered the HDL-anti-inflammatory and reduced lesions by 79% without altering plasma cholesterol or HDL-cholesterol levels (10). Similarly adding D-4F to the drinking water of apoE null mice converted their HDL from pro-inflammatory to anti-inflammatory and atherosclerotic lesions were decreased by 75% (10).

Van Lenten et al. found that influenza A infection in mice caused their HDL to become pro-inflammatory (i.e. instead of inhibiting LDL-induced monocyte chemotactic activity the HDL actually increased LDL-induced monocyte chemotactic activity) (11). Influenza A pneumonia in LDL receptor null mice that had been on a Western diet caused HDL to become profoundly pro-inflammatory and also increased macrophage traffic into the aortic arch and innominate arteries (12). Treatment of the mice with D-4F dramatically reduced the inflammatory components of the viral pneumonia, reduced viral titers, restored the HDL to anti-inflammatory, and dramatically decreased macrophage traffic into the arteries (12).

Ou et al. found that 4F synthesized from all L-amino acids (L-4F) and injected into LDL receptor null mice on a Western diet dramatically improved arterial vasoreactivity (13). Similar

findings were noted in a mouse model of sickle cell disease, which has a profound impairment in arterial vasoreactivity (13).

Oral administration of D-4F to apoE null mice resulted in the formation of pre- β HDL, reduced lipoprotein lipid hydroperoxides, increased anti-oxidant enzyme activity in HDL (paraoxonase activity), improved HDL-mediated cholesterol efflux and promoted reverse cholesterol transport from macrophages (14).

Li et al. administered D-4F orally or by injection to apoE null mice on a Western diet in which a segment of the inferior vena cava had been inserted into the carotid artery. D-4F treatment dramatically reduced lesion formation including plaque lipid and macrophage content in this accelerated vein graft model (15). While there was a significant reduction in macrophage content in previously established aortic lesions there was no significant reduction in lesion area. The authors thought that there might be a differential effect on developing compared to already established lesions. However, they acknowledged that they only treated the mice for 4 weeks during the postoperative period in a model with severe hyperlipidemia (i.e. apoE null mice on a Western diet) (15).

Administration of oral D-4F to monkeys reduced lipoprotein lipid hydroperoxides, improved HDL anti-inflammatory properties, improved the ability of the HDL to mediate cholesterol efflux, and reduced LDL's ability to stimulate monocyte chemotactic activity (16,17).

Oral administration of D-4F and pravastatin to apoE null mice at doses that were found to be ineffective by themselves resulted in a remarkable synergy that increased HDL-cholesterol levels, apoA-I levels, paraoxonase activity, rendered HDL anti-inflammatory, prevented lesion formation in young and caused regression of established lesions in old mice (17). Lesion macrophage content was reduced by 79%. The combination also increased intestinal apoA-I synthesis by 60% in these mice (17).

Ou et al. reported that oral administration of D-4F improved arterial vasoreactivity in LDL receptor null mice fed a Western diet (18). They also found that D-4F prevented the diet-induced thickening of the arterial wall (18). Moreover, they found that D-4F treatment reduced pre-existing arterial wall thickness in this mouse model implying that the treatment caused lesion regression. Treatment of the mice with D-4F rendered HDL anti-inflammatory unless the mice also lacked apoA-I. In the absence of apoA-I, D-4F significantly improved arterial vasoreactivity but did not reduce arterial wall thickness. *In vitro* 4F reduced collagen expression by endothelial cells (18). Interestingly D-4F treatment reduced the association of myeloperoxidase with apoA-I in HDL *in vivo* but not *in vitro* (18).

ApoA-I Mimetic Peptides in Sepsis

Gupta et al. reported that L-4F blocked the binding of bacterial lipopolysaccharide (LPS) to its binding protein and decreased the binding of LPS to cultured endothelial cells and reduced cytokines, chemokines and adhesion molecules including VCAM-1 expression. (19). In Sprague-Dawley rats VCAM-1 expression was significantly reduced after intravenous injection of LPS and intraperitoneal injection of L-4F (19).

ApoA-I Mimetic Peptides in a Rat Model of Diabetes

Kruger et al. found that D-4F injection into Sprague-Dawley rats made diabetic by administration of streptozotocin dramatically decreased superoxide levels, reduced the levels of circulating endothelial cells, decreased endothelial cell fragmentation, and restored arterial vasoreactivity (20). The mechanism appeared to involve the induction of arterial heme oxygenase-1 and extracellular superoxide dismutase (20).

ApoA-I Mimetic Peptides in LDL Receptor Null Mice with Cognitive Dysfunction Induced by a High Fat Diet

Feeding a Western diet to LDL receptor null mice for 6 – 8 weeks resulted in a decline in cognitive function that was significantly ameliorated by adding D-4F to their drinking water but not the inactive control peptide scrambled D-4F (21). The improvement in cognitive function was achieved without any significant change in total cholesterol, apoB containing lipoprotein-cholesterol, HDL-cholesterol, or triglycerides.

As noted above (12) feeding a Western diet to LDL receptor null mice resulted in macrophage infiltration into the large and medium sized arteries. In atherosclerosis the macrophages are derived from blood monocytes that cross the endothelium in response to the production of chemokines such as MCP-1 where they convert into macrophages in the subendothelial space of the vessel (22). These macrophages are located between the endothelial monolayer on the luminal side of the vessel and the smooth muscle cells in the media of the vessel. Examination of the brains of the mice on the Western diet revealed a dramatic increase in the production of chemokines such as MCP-1 and MIP-1 α in brain arterioles (21). Arterioles are arterial vessels with luminal diameters between 10 and 100 μ m. A normal human coronary artery has a luminal diameter of 1,000 to 3,000 μ m. The hyperlipidemia-induced increase in MCP-1 and MIP-1 α was accompanied by a dramatic increase in brain arteriole associated macrophages (microglia) (21). However, unlike the aorta where the macrophages were found between the endothelial and smooth muscle cell layers of the artery, in the brain arterioles the macrophages were always found intimately associated with the brain arteriole on the adventitial side of the vessel, often incorporated into the basal lamina of the vessel (21). While the Western diet dramatically increased the number of macrophages intimately associated with the brain arterioles there was no increase in brain macrophages not associated with arterioles. However, there was a dramatic increase in the chemokines MCP-1 and MIP-1 α associated with neuronal cells that were found around the brain arterioles of the mice on the Western diet (21). It has been reported that non-vascular brain cells also produce these chemokines and have receptors for these chemokines and it is thought that they may regulate synaptic transmission and neuronal survival (23,24). The percent of brain arterioles and neuronal cells around the arterioles that expressed MCP-1 and MIP-1 α were significantly reduced by the addition of D-4F to the drinking water of the mice (21). It was concluded that with the induction of hyperlipidemia by the Western diet the brain arterioles became foci of inflammation and that D-4F reduced arteriole inflammation as it had reduced inflammation in the aorta and innominate arteries (12). As a result of this anti-inflammatory action of D-4F on the arterioles there was decrease in chemokines such as MCP-1 and MIP-1 α that diffused from the arterioles to the surrounding neuronal cells with a consequent improvement in cognitive function without an alteration in plasma lipids or blood pressure (21).

ApoJ Mimetic Peptides and Tetrapeptides in Mouse Models of Atherosclerosis and in Monkeys

Using the artery wall coculture system it was found that another HDL-associated apoprotein, apoJ, had potent ability to inhibit the LDL-induced monocyte chemotactic activity in a co-incubation (24). Thus, apoJ was more potent in its ability to sequester oxidized lipids than was apoA-I since apoA-I did not inhibit LDL-induced monocyte chemotactic activity in a co-incubation (3). Navab et al. (25) identified 17 potential G* amphipathic helices in the mature apoJ protein. They synthesized 7 of these sequences and tested them in the artery wall cell culture model. Six of the 7 were effective in decreasing LDL-induced monocyte chemotactic activity but only 2 were as effective as the intact apoJ protein and only one inhibited lesion formation in apoE null mice (25). This sequence synthesized from all D-amino acids contained

residues 113 – 122 in apoJ and was designated D-[113-122]apoJ. After an oral dose D-[113-122]apoJ more slowly associated with lipoproteins and was cleared from plasma much more slowly than D-4F. Oral administration of D-[113-122]apoJ significantly improved the ability of plasma to promote cholesterol efflux and rendered the pro-inflammatory HDL of apoE null mice anti-inflammatory for up to 48 hours after a single dose. Oral administration of D-[113-122]apoJ reduced aortic atherosclerosis in apoE null mice by about 70%. In monkeys oral D-[113-122]apoJ rapidly reduced lipoprotein lipid hydroperoxides and improved HDL anti-inflammatory properties.

The peptides described above contain class A amphipathic helices (4F and 5F) or G* amphipathic helices [113-122]apoJ. To determine if a helix is required for biologic activity tetrapeptides with amphipathic and zwitterionic properties were synthesized that were too small to form a helix (26). Oral administration of a tetrapeptide containing only 4 amino acid residues (KRES) that is too small to form an amphipathic helix reduced lipoprotein lipid hydroperoxides, increased paraoxonase activity, increased HDL-cholesterol levels, rendered HDL anti-inflammatory, and reduced atherosclerosis in apoE null mice (26). This peptide was equally orally effective when synthesized from either D- or L-amino acids. Changing the order of the 2 central amino acids (KRES to KERS) resulted in the loss of all biologic activity. Another tetrapeptide FREL shared many of the physical-chemical properties of KRES and was biologically active in mice and monkeys when synthesized from either L- or D-amino acids and administered orally (26).

ApoE Mimetic Peptide

Gupta et al. synthesized a peptide with the receptor binding domain of apoE covalently linked to the class A amphipathic helical peptide 18A (27). This dual domain peptide was designated Ac-hE-18A-NH₂ to indicate that the carboxy terminus of the apoE binding domain was acetylated and the amino terminus of the class A amphipathic helix domain was aminated. Eighteen hours after a single intravenous bolus (15 mg/kg) of the peptide plasma cholesterol levels in Watanabe heritable hyperlipidemic rabbits were reduced from 562 ± 29 mg/dL to 288 ± 22 mg/dL (27). Plasma lipid hydroperoxide levels were significantly reduced and the HDL-associated anti-oxidant enzyme paraoxonase showed a 5-fold increase in activity (27). Associated with these changes were significant reductions in the formation of superoxide anion, a scavenger of nitric oxide in the arteries of the rabbits (27).

Mechanisms of Action of Peptide Mimetics of Apolipoproteins

The biologically active peptides described above share a number of common characteristics. They all bind lipids and lipoproteins. Almost all of the peptides associated with HDL, however, the rate of association and dissociation of the peptides from HDL varied considerably (8,9, 25,26). Administration of all of the peptides leads to a reduction in lipoprotein lipid hydroperoxides. Indeed adding most of these peptides to plasma *in vitro* followed by separation of the plasma by FPLC resulted in a reduction in lipoprotein lipid hydroperoxides that was not seen when scrambled inactive peptides were added (8,25,26,28). Concomitant with a decrease in HDL-lipid hydroperoxides was an increase in the activity of the anti-oxidant HDL-associated enzyme paraoxonase and an improvement in the ability of HDL to inhibit LDL-induced monocyte chemotactic activity (8,25,26,28). Thus, one mechanism of action appears to involve the removal of oxidized lipids from lipoproteins. Bielicki and Forte published that lipid hydroperoxides inhibit LCAT (29) and Forte and colleagues reported that the activities of LCAT, paraoxonase, and platelet activating factor acetylhydrolase in atherosclerosis susceptible mice correlated with plasma levels of oxidized lipids (it was concluded that the oxidized lipids inhibited the activities of these enzymes) (30). One common mechanism for

the action of these peptides may be the binding and removal of oxidized lipids that inhibit the anti-oxidant enzymes associated with HDL, thus releasing the enzymes from inhibition (25).

As noted above, the peptides 4F, 5F, and [113-122]apoJ bind these oxidized lipids such that they are effectively sequestered in a co-incubation with LDL and human artery wall cells. In contrast the binding of these lipids to apoA-I and the tetrapeptides appears to be less avid such that these peptides must be removed from the incubation after binding the oxidized lipids or the reaction proceeds as if the peptides were not present. These data suggest that the binding constant for the oxidized lipids bound by 4F, 5F, [113-122]apoJ may be orders of magnitude different from that of apoA-I and the tetrapeptides (i.e. the affinity of oxidized lipids for 4F, 5F, and [113-122]apoJ is orders of magnitude greater than it is for apoA-I and the tetrapeptides).

Another feature of some of the peptides (but not all) relates to their ability to promote cellular cholesterol efflux and reverse cholesterol transport (14,16,25). Reddy et al. demonstrated that cholesterol loading of human aortic endothelial cells increased their production of reactive oxygen species and greatly enhanced LDL-induced monocyte chemotactic activity all of which were greatly ameliorated by treatment with an apoA-I mimetic peptide (31).

Tang et al. found that 2F and 4F promoted ABCA1-dependent cholesterol efflux and competed for apoA-I binding to ABCA1-expressing cells, blocked covalent cross-linking of apoA-I to ABCA1 and inhibited ABCA1 degradation (32). These peptides stimulated JAK2 autophosphorylation and inhibition of JAK2 greatly reduced peptide-mediated cholesterol efflux, peptide binding to ABCA1-expressing cells, and peptide cross linking to ABCA1 (32). In contrast apoA-I and the peptides stabilized ABCA1 protein even in the absence of JAK2 (32).

The enhanced formation of pre- β HDL after administration of 4F may relate to its ability to separate cholesterol from phospholipid (33). As a result the 4F peptide may stimulate the normal cycle by which lipid free apoA-I dissociates from mature HDL to form pre- β HDL.

Anantharamaiah and colleagues used class A amphipathic peptides with 3 phenylalanine residues on the hydrophobic face to determine the characteristics of the peptides that were required for biologic activity. They compared 3F-2 and 3F¹⁴ and found that 3F-2 inhibited lesions in apoE null mice while 3F¹⁴ did not (34). 3F-2 preferentially associated with HDL while 3F¹⁴ preferentially associated with apoB-containing particles (34) confirming the importance of HDL-association in the mechanism of action of these peptides.

Summary

Many of the properties of apoA-I and apoJ and some of the properties of apoE can be mimicked by small peptides. In some cases these peptides appear to bind oxidized lipids better than apoA-I and in all cases studied the peptides improved HDL function. These peptides have resulted in improvement in multiple vascular beds suggesting that a strategy of using peptides that mimic apoproteins associated with HDL may prove to be a successful strategy for treating a number of vascular diseases.

Acknowledgements

This work was supported in part by US Public Health Service grants HL-30568 and HL-34343 and the Laubisch, Castera, and M.K. Grey Funds at UCLA.

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