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HDL apolipoprotein-related peptides in the treatment of atherosclerosis and other inflammatory disorders

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Abstract

Elevations of HDL levels or modifying the inflammatory properties of HDL are being evaluated as possible treatment of atherosclerosis, the underlying mechanism responsible for most cardiovascular diseases. A promising approach is the use of small HDL apoprotein-related mimetic peptides. A number of peptides mimicking the repeating amphipathic α -helical structure in apoA-I, the major apoprotein in HDL, have been examined in vitro and in animal models. Several peptides have been shown to reduce early atherosclerotic lesions, but not more mature lesions unless coadministered with statins. These peptides also influence the vascular biology of the vessel wall and protect against other acute and chronic inflammatory diseases. The biologically active peptides are capable of reducing the pro-inflammatory properties of LDL and HDL, likely due to their high affinity for oxidized lipids. They are also capable of influencing other processes, including ABCA1 mediated activation of JAK-2 in macrophages, which may contribute to their anti-atherogenic function. The initial studies involved monomeric 18 amino acid peptides, but tandem peptides are being investigated for their anti-atherogenic and anti-inflammatory properties as they more closely resemble the repeating structure of apoA-I. Peptides based on other HDL associated proteins such as apoE, apoJ and SAA have also been studied. Their mechanism of action appears to be distinct from the apoA-I based mimetics.

Keywords

apoproteins; mimetic peptides; apoA-I; HDL; atherosclerosis; inflammation

Epidemiologic analysis of cardiovascular disease development and outcomes indicates that plasma HDL levels are important negative risk factors [1]. The basis for much of cardiovascular disease is underlying atherosclerosis and its complications. Much of the evidence supporting the atheroprotective influence of HDL derives from animal models of atherosclerosis, such as the effects of the transgenic overexpression of human apolipoprotein A-I (apoA-I) in mice [2]. The large majority of the apoA-I in the plasma is associated with lipoproteins and is the major apoprotein of HDL. There have been many proposed mechanisms by which HDL/apoA-I may serve to protect against the development of atherosclerosis [3, 4]. These include anti-inflammatory and anti-oxidative effects, promotion of reverse cholesterol transport (i.e. the transport of cholesterol from the atherosclerotic lesions to the liver), enhancement of endothelial nitric oxide synthase and anti-thrombotic effects. Elevation of HDL levels in humans is an area of intense research [5]. Recent attempts to administer apoA-I to atherosclerotic patients have yielded suggestive encouraging results [6, 7], though the controls and endpoints are not yet compelling.

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ApoA-I is a 243 amino acid protein, making repeated administration of the protein for a chronic inflammatory disease such as atherosclerosis daunting. ApoA-I is encoded by a gene with four exons, but only the codons derived from exons three and four direct the sequence of the mature protein; exon three encodes the N-terminal 43 amino acids and exon four encodes the remainder of the protein [8]. The exon four encoded amino acids are made up of a series of 10 repeating amphipathic α -helices, eight of which are 22 amino acids in length and the other two consisting of 11 amino acids. In an attempt to understand the basis for the lipid binding properties of apoA-I, Segrest and colleagues studied the lipid binding properties of each of the amphipathic helices in the protein and from this developed a model 18 amino acid amphipathic helical peptide which was not identical in sequence to any of the helices of apoA-I, but nevertheless resembled their average mean biophysical properties [9]. This peptide, referred to as 18A, has been the basis for the development of experimentally valuable apoA-I mimetic peptides.

Physical properties of apoA-I mimetic peptides

The sequence of the 18A peptide is DWLKAIFYDKVAEKLKEAF. The 18A peptide describes an amphipathic α -helix with segregation of the hydrophobic residues to the non-polar face and the hydrophilic residues to the polar face (Figure). Several lysine residues are positioned at the interface between the polar and nonpolar faces and negatively charged amino acids in the center of the polar face. This type of amphipathic helix has been termed a class A helix.

The lipid binding properties of mimetic peptides are assessed by their ability to solubilize phospholipids and form discoidal phospholipid particles. Neutralizing the charges at the N-terminus of the 18A peptide by acetylation and the C-terminus by amidation increases the peptide's helicity, its self-association capacity, as well as its lipid binding affinity [10, 11]. In complexes with lipids, the hydrocarbons in the side chain of the lysine residues in the class A amphipathic helices interact with the acyl chains of the phospholipids while the NH_3^+ group extends toward the polar face of the helix in a process known as "snorkeling" [12]. This improves lipid binding of the peptide by allowing it to penetrate deeper into the phospholipid bilayers of the particle. The importance of the length of the hydrophobic portion of the side chain of the lysine residues at the interface of the polar and nonpolar faces is indicated by substituting these residues with an amino acid having a shorter side chain, i.e. homoaminoalanine residues, which reduces the lipid binding of the peptide [12]. Even though having the negatively charged residues glutamate or aspartate at these interfacial positions may improve the peptide's helicity, such peptides have reduced lipid binding propensity than those with lysines in this position [13]. As with apoA-I, the peptide forms discoid particles when associated with phospholipids. When the peptide-phospholipid complex is modeled based on NMR data, the peptides are aligned in a head to tail configuration perpendicular to the axis of the phospholipid acyl tails [14].

Variants of the 18A peptide have been generated and the properties of several of these variants are well reviewed by Anantharamaiah GM et al [15]. The variants are all acetylated and amidated and are named based on the number and in some cases the location of phenylalanines on the hydrophobic surface of the amphipathic peptide. Thus the acetylated and amidated 18A peptide is referred to as 2F and possesses two phenylalanine residues at positions 6 and 18 (see Figure 1). The substitution of leucine residues at positions 3 and 14 with phenylalanine residues within 2F generates the peptide 4F, an 18 amino acid helical peptide containing phenylalanines at positions 3, 6, 14 and 18. 4F is the most studied apoA-I mimetic peptide. The additional phenylalanine residues in 4F are at the center of the peptide's non-polar face, whose hydrophobicity is thereby increased. These phenylalanine residues, which compared to leucine residues are rich in slightly polar π electrons, allow the

peptide to associate with potentially pro-inflammatory oxidized phospholipid [15]. Other phenylalanine substitution variants that have been studied are 5F (with phenylalanines at positions 6, 11, 14, 17, and 18), 6F (with phenylalanines at positions 6, 10, 11, 14, 17 and 18) and 7F (with phenylalanine residues at positions 3, 6, 10, 11, 14, 17 and 18). When assayed in vitro for ability to inhibit monocyte chemotaxis (see next section) and activation of LCAT, 2F and 7F are the least bioactive [16] (Table 1).

An instructive set of four peptide variants, each containing three phenylalanine residues, has been studied: 3F-1 (with phenylalanines at positions 6, 10 and 18), 3F-2 (with phenylalanines at positions 10, 14 and 17), 3F-3 (with phenylalanines at positions 3, 6 and 18) and 3F-14 (with phenylalanines at positions 6, 14 and 18). All have similar secondary structure and physical properties [17, 18]. The first two of these variants are bioactive with respect to the ability to remove hydroperoxides from LDL and inhibit monocyte chemotaxis, while the last two are not [17]. The bioactive peptides (3F-1 and 3F-2) are predicted to have larger hydrophobic faces as compared to the inactive peptides (3F-3 and 3F-14). This potentially explains the differences in peptide interaction with the acyl chains of the phospholipid layer [17, 18]. 3F-2 is also more helical than the other peptides, including 4F. When assaying the capacity to solubilize POPC, 3F-3 and 3F-14 are more effective than 3F-1, 3F-2 and 4F; this capacity is therefore not correlated with biological activity. Tryptophan residues in 3F-1, 3F-2, and 4F are less motionally restricted than is the case for 3F-3 and 3F-14 [17]. Lack of tryptophan motion restriction is correlated with peptide biological activity and it has been hypothesized that tryptophan motion allows solubilization of polarized pro-inflammatory moieties within peptide-associated lipoproteins.

Anti-inflammatory properties of apoA-I mimetic peptides

The monocyte chemotactic assay has been very valuable in characterizing the anti-inflammatory properties of the mimetic peptides, though the assay has been established in only a few laboratories. In this assay, human endothelial cells are cocultured atop smooth muscle cells to resemble an artery. The cocultures are incubated in the presence of LDL as well as peptides, apoproteins, or other lipoproteins. The culture medium is then removed, fresh media added, and this media transferred to one side of a culture chamber divided by a permeable membrane, with monocytes added to the other side. Any coculture-related lipoprotein oxidation stimulating the production of monocyte chemotactic protein (MCP-1) by the endothelial cells will lead to the transmigration of monocytes. Exogenous addition of oxidized long chain polyunsaturated fatty acids to the LDL added to the coculture converts it to a more proinflammatory lipoprotein (i.e. increases the production of MCP-1 and the transmigration of monocytes) [19]. The *de novo* oxidation of LDL probably arises from endothelial cell-derived lipoxygenases; an anti-sense to 12-lipoxygenase reduces the oxidative modification of LDL and thus attenuates the lipoprotein's proinflammatory activity [20]. When HDL from healthy individuals is added to the coculture system, the pro-inflammatory effect of LDL is attenuated. In contrast, the coinubation of LDL with lipid-free apoA-I does not attenuate LDL-induced monocyte chemotaxis [20, 21], although preincubation of cells with apoA-I and removal of the apoprotein prior to the addition of LDL results in attenuation of LDL-induced monocyte chemotaxis [20].

The monocyte chemotactic assay allows for the characterization of the pro- and anti-inflammatory properties of lipoproteins derived from patients. Unlike HDL from normal healthy individuals, HDL obtained from patients with acute coronary disease is not as effective in inhibiting LDL-induced chemotaxis, indicating that the HDL from patients has a higher inflammatory index than normal HDL [22]. Thus, this assay has been particularly valuable in defining the functional activity of HDL leading to appreciation of the fact that

more than HDL level has to be taken into account in evaluating the anti-inflammatory drive of HDL.

The apoA-I mimetic peptides also have the capacity to attenuate the inflammatory properties of LDL in the co-culture assay. But in contrast to apoA-I [20, and 21], the coincubation of LDL with mimetic peptides has an anti-inflammatory effect [16]. This difference in the properties of apoA-I and the mimetic peptides suggests that the mimetic peptide sequesters the LDL modifier (i.e. lipid hydroperoxides) more avidly than does apoA-I, thereby preventing interaction of the proinflammatory lipids with LDL. Such a suggestion received strong validation in a recent study by Van Lenten and colleagues, who demonstrated using surface plasmon resonance that some of the mimetic peptides bind oxidized lipids (oxidized fatty acids, phospholipids and sterols) with an affinity that is several orders of magnitude higher than that of apoA-I [23]. Interestingly there was a strong correlation between the binding affinity for oxidized lipids and in vivo bioactivity against atherosclerosis; e.g. 3F-14, which is not bioactive in vivo, does not bind oxidized lipids with high affinity, while 3F-2 is effective in both respects. The ability of active mimetic peptides to bind and sequester oxidized lipids may be an important contributor to the atheroprotective action of these monomeric mimetic peptides.

The 18A based mimetic peptides contains 4 lysine residues and when synthesized with L amino acids they are potentially susceptible to tryptic hydrolysis. In order to overcome this instability, especially for oral treatment, several of the apoA-I mimetic peptides have been synthesized with D-amino acids. The D-amino acid peptides have similar chemical, biophysical, and biological properties to those peptides synthesized from natural L-amino acids [24-26]. This suggests that the stereochemistry of these peptides is not a critical element of their bioactivity. From this one can conclude that the atheroprotective action of these peptides is unlikely to be attributable predominantly to a direct interaction with the active center of one or more enzymes which are normally stereochemically specific. However there is at least one example where the biophysical properties of the two stereoisomers differ significantly. This relates to the binding affinity for 20(S)hydroxycholesterol which is 10 fold higher for L4F than D4F [23].

In addition to the apoA-I mimetic peptides' anti-inflammatory ability to bind oxidized lipids, other biological properties could influence their athero-protective effects. These peptide properties include remodeling of HDL, changes in the expression and activity of antioxidative enzymes, and the promotion of reverse cholesterol transport [27, 28].

The peptides may also exert anti-inflammatory effects on macrophages. The binding of apoA-I to ABCA1 promotes the autophosphorylation of JAK2 and activation of STAT3, which promotes an anti-inflammatory phenotype in macrophages [29]. This anti-inflammatory effect is independent of ABCA1 mediated lipid efflux. The 2F and 4F peptides also stimulate the autophosphorylation of JAK2 in an ABCA1 dependent process [26]. The effect of the peptides on STAT3 activation and inflammatory gene expression has not yet been examined. In addition, the observation that ABCA1 deficient macrophages have increased expression of pro-inflammatory genes that is reversed by decreasing cellular cholesterol with methyl- β -cyclodextrin [30] suggests that possibility that peptide-mediated cholesterol efflux may also contribute to decreasing macrophage inflammation.

Cholesterol efflux properties of apoA-I mimetic peptides

Several of the 18A-based peptides have been demonstrated to promote cholesterol efflux from cholesterol loaded cells in culture (Table 1). This has been demonstrated in J774 macrophage-like cells treated with cAMP to upregulate ABCA1 expression and in ABCA1 transfected HeLa and BHK cells. Interestingly in ABCA1 expressing cells, 2F, D2F and 4F

are equally efficient in promoting cholesterol efflux, at levels comparable to apoA-I [26]. This is attributable primarily to their binding to ABCA1, leading to stabilization of the transporter. It is of interest that the stereochemistry of the 2F peptide is indifferent for its interaction with ABCA1. There also appears to be significant interaction of the peptides with the cell surface that is independent of ABCA1 [26] indicating that at least part of the lack of stereospecificity of the peptides for cholesterol efflux may be due to their high affinity for the cell membrane lipids.

HDL from apoE deficient mice treated with a single dose of D4F promotes increased cholesterol efflux from cholesterol labeled monocyte macrophages in vitro [31]. Additionally, the oral administration of D4F promotes in vivo reverse cholesterol transport as monitored by the movement of radiolabeled cholesterol from cholesterol loaded J774 cells injected into the peritoneal cavity into the blood and feces of peptide treated mice. In the same study, D4F administration reduced lipid peroxide in VLDL/IDL, LDL and HDL with an increase of these oxidized lipids in the pre- β HDL.

Given the selective biological effects of the 3F peptide variants (3F-1, 3F-2, 3F-3, 3F-14), it would be of great interest to have information on their effects on cholesterol efflux and reverse cholesterol transport.

HDL association of apoA-I mimetic peptides

Treatment of mice or isolated plasma with apoA-I mimetic peptides has been shown to modify the properties of the HDL. The peptides decrease the inflammatory properties of HDL as measured in the monocyte chemotaxis assay [31] and remodels the HDL [31-34]. This remodeling results in the production of pre- β HDL particles containing apoA-I. However, the concentration of peptide required to remodel HDL is higher than that needed to influence atherosclerosis. Thus this pathway is unlikely to be important for their anti-atherogenic functions [28].

The focus of peptide actions on HDL structure and function would lead one to expect that these peptides readily associated with HDL. However there is some controversy about the avidity of the 4F peptide for HDL [35, 36]. The resolution of this controversy is impeded by the inherent difficulty of monitoring the peptide in vivo. Unfortunately no antibody is currently available to detect these peptides. The studies that have been conducted have employed 4F peptides labeled with radioactivity or with such tags as anthranilic acid or biotin. In our in vivo studies we have employed biotinylated peptides and demonstrate that the biotinylated peptide in apoE deficient mouse plasma is not associated with the major HDL peak 2 hours after intraperitoneal injection but is predominantly found in a smaller particle that does not contain apoA-I [35]. A very similar distribution is observed simply by incubating 4F with plasma ex vivo. Furthermore the same distribution is observed when the plasma contains no distinct HDL peak, as in mice deficient in both apoE and apoA-I. In addition, we have not been able to demonstrate a high affinity interaction of the biotinylated peptide with HDL by surface plasmon resonance (SPR). It is possible that biotinylation changes the properties of the peptide. At least one in vitro property of the peptide is not altered by biotinylation; the biotinylated and non-biotinylated peptides promote cholesterol efflux from macrophages equivalently (unpublished data). The lack of HDL binding by the monomeric 4F peptide are in contrast to a biotinylated 4F dimer separated by a proline residue which associates with HDL in apoE^{-/-} plasma and binds the HDL with high affinity by SPR [35]. Further studies are clearly required to resolve these issues, including examining if modifications of the peptides occur in vivo. It is clear from the remodeling studies of mimetic peptides that they do interact with HDL at least transiently in vitro.

Tandem apoA-I mimetic peptides

The first of the tandem peptides based upon the 18A theme is 37pA which is constituted of two monomeric 18A amphipathic peptides linked by single proline residue. The tandem peptide promotes cholesterol efflux almost as well as monomeric 2F [10, 26] and also stabilizes ABCA1 [26]. Similar to 2F [26], the L- and D-amino acids isomers of 37pA are equally effective in promoting both ABCA1 dependent and independent cholesterol efflux [24]. A related peptide with alanine linking the two 18A monomers instead of proline also promotes cholesterol efflux [37]. An asymmetric tandem peptide, designated 5A, has been studied in which the 18A monomer is linked via proline residue to a modified 18A monomer in which the hydrophobic residues at positions 3, 6, 10, 14, and 18 were replaced by an alanine residue, thus reducing the hydrophobicity of the second helix in the tandem peptide [38]. The rationale for designing the asymmetrical peptide is that the neighboring helices of apoA-I are not identical in physical properties. Interestingly if the 3, 6 and 10 substitutions were made in the first helix instead of the second helix, the properties of the asymmetric peptide (i.e. 5A-2 peptide) is quite different than the peptide in which the alanine substitutions had been made in helix 2. 37pA and 5A peptides were capable of solubilizing lipid, though their relative efficacy depends on the nature of the lipid with 5A solubilizing DMPC (dimyristoylphosphatidyl choline) better than 37pA, while the reverse is the case when the lipid is a mixture of phospholipids including some negatively charged phospholipids. The alanine residues of these peptides do not penetrate the lipid acyl chains as deeply as the hydrophobic residues for which they are substituting. 5A-2 is barely effective at solubilizing the phospholipids. Also the comparative ABCA1 dependent cholesterol efflux capacity of these peptides depends upon the concentration of peptide employed. 37pA is more effective than 5A at quite low concentration but the reverse is true at higher concentrations of peptide. Other relevant properties of the 5A peptide are noted with red blood cells and endothelial cells. While 37pA at high concentrations is capable of lysing red cells (about 20 to 30% at 10 to 15 μ mol), 5A was not cytotoxic [38]. 37pA is not cytotoxic in nM concentration range [39]. 5A has also recently been shown to attenuate the pro-inflammatory reaction induced by TNF α in human carotid artery endothelial cells (reduced VCAM1 and ICAM1 expression) and this attenuation is ABCA1 dependent [40]. Whether this involves STAT 3 activation remains to be determined. In our laboratory we have studied a series of 4F-based tandem peptides in which two 4F monomers are linked to one another by either a proline residue (4F-Pro-4F), an alanine residue (4F-Ala-4F), or a seven amino acid inter helical sequence identical to the seven amino acids derived from apoA-I sequence between helices 4 and 5 of the intact apoprotein (4F-IHS-4F) [33]. These three tandem peptides are active in the remodeling of HDL, effectively depleting apoA-I but not apoA-II from HDL. They are also more effective than monomeric 4F in promoting cholesterol efflux from cholesterol loaded J774 macrophages by an ABCA1 dependent process. The 4F-Pro-4F peptide binds to HDL in vivo as well as ex vivo [35].

Other apoA-I related peptides

Though not strictly mimetic peptides in as much as they are derived directly from the sequence of apoA-I, the properties of several apoA-I sequence derived peptides have been studied. Helices 1 (residues 44 to 65) and 10 (residues 220-241) are the most lipophilic of the apoA-I helices. These single 22-amino acid helices are quite ineffective at promoting cholesterol efflux [41]. However when each of these peptides is directly linked with helix 9 (residues 209-219), an 11 amino acid helix, to generate 33 amino acid peptides, the 1/9 peptide and the 9/10 peptide were as effective at the promotion of ABCA1 dependent cholesterol efflux as intact apoA-I, at least on an equivalent weight basis, and stabilized ABCA1 on the cell membrane. There is a degree of specificity in that 33 amino acid peptides composed of helices 1/3, 2/9 and 4/9 are not active in these efflux assays.

Reversing the order of helices 9 and 10 to generate the 10/9 peptide attenuates the ability of the peptide to promote efflux, despite having the same amino acid composition and lipid binding activity as the 9/10 peptide. On the other hand, the 9/1 reversed peptide efficiently promoted cholesterol efflux. This led to the suggestion that the lipid efflux capacity of the peptides is not correlated with its hydrophobicity or lipid binding capacity, but rather with the distribution of acidic residues along the polar face of the helices.

ApoJ derived peptides

ApoJ or clusterin is a chaperone protein which may have either an intracellular or extracellular function [42]. Its gene encodes a 449 amino acid protein. As a secreted protein, it has a 22 amino acid signal peptide. The mature protein undergoes a proteolytic cleavage, producing α - and β -chains, which are linked as a heterodimer by five disulfide bonds. The protein is also glycosylated at multiple sites. As an apolipoprotein, apoJ is found in a subset of dense HDL particles containing apoA-I and paraoxonase (PON) [43]. The ratio of apoJ/PON is higher in individuals at risk of future clinical cardiovascular disease [44].

ApoJ has a large number of “potential” amphipathic G* helices. Navab and colleagues have synthesized peptides corresponding to seven of these helices. Six of these peptides are active in protecting against LDL-induced monocyte migration in the monocyte chemotactic assay, with two peptides being as protective as full-length apoJ [45]. The active peptides correspond to residues 113-122 and 336-357. These peptides are not helical in saline, but the helicity increases in the presence of lipids or detergents (e.g. in 1% SDS, the apoJ 113-122 peptide has a helicity of 31%). When apoE deficient mice are acutely treated with apoJ 113-122 peptide, the treated plasma exhibits increased cholesterol efflux capacity. Also PON is increased and lipid peroxides levels are reduced in the plasma of monkeys acutely treated with the apoJ peptide. A similar reduction in lipid peroxides was observed following *in vitro* incubation of plasma from apoE deficient mice with the apoJ peptide. This was also associated with an increase in PON activity.

ApoE related peptides

ApoE is a multifunctional protein [46]. The mature protein is 299 amino acids in length and in humans there are three isoforms of the protein with different functional properties. ApoE is also well known to be a modulator of lipoprotein metabolism, atherosclerosis and immune function. ApoE also functions in the nervous system, especially the central nervous system. Astrocytes are the main cells in the central nervous system expressing apoE where it is thought to be involved in the transfer of cholesterol from the astrocytes to neurons. As shown by extensive work by Mahley and others, the apoE4 isoform is a well-established risk factor for the development of Alzheimer's disease [47].

ApoE is found in a subset of HDL particles, in VLDL and chylomicron remnants. It functions as an important ligand for the uptake of these lipoproteins by the LDL receptor and related members of this family of receptors. The ligand active portion of the protein has been mapped to residues 141-150, which colocalizes with a major heparin binding site. A peptide composed of a dimer of residues 141-155 with an N-terminal tyrosine residue (designated Y (141-155)₂) binds to the LDL receptor and upon acetylation of its N-terminus also binds to essentially all lipoproteins [48]. Acute injection of the acetylated peptide, but not the non-acetylated peptide, into apoE^{-/-} mice promotes the clearance of the VLDL and IDL.

A chimeric protein containing apoE residues 141-150 linked to one copy of 18A (Ac-hE-18A-NH₂) also binds VLDL and LDL and promotes the uptake of these lipoproteins by cells [49, 50]. When this peptide is administered to Watanabe heritable

hypercholesterolemic rabbits as a single bonus, it results in a 50% decline in total plasma cholesterol, a decline in plasma lipid peroxide levels and an increase in HDL associated PON activity [51]. It also suppresses the production of superoxide by the arteries of peptide treated rabbits.

Like apoA-I, apoE promotes cholesterol efflux. This activity can be duplicated by the C-terminal domain of apoE, residues 222 to 299, which has the same efficiency as intact apoE and apoA-I [52]. Also like apoA-I, its lipid efflux activity is ABCA1 dependent. Further truncation of the C-terminal domain indicates that sequences containing adjacent class A and class G amphipathic helices are important for the efflux activity of the C-terminus of apoE. Recently Bielicki and colleagues have modified this latter domain, generating a 25 amino acid synthetic peptide, designated AT 1-5261 [53]. It differs from the parent peptide in several residues, so that the nonpolar face has fewer residues, 9 instead of 11, but is more hydrophobic. The polar face is more acidic with the substitution of one of its residues by an additional glutamic acid. So the polar face now has six glutamic acid residues in contrast to the five of the parent peptide. It exhibits good cholesterol efflux capacity, with a similar V_{max} to intact apoA-I but a significantly lower K_m (four times lower). It may be used either as a free peptide or in a complex with phosphatidylcholine. The complex also promotes cholesterol efflux that is partially ABCA1 dependent, though it has a relatively high K_m in this state (20 times higher). A single injection of this peptide is capable of promoting in vivo reverse cholesterol transport.

Serum amyloid A

Serum amyloid A (SAA) is a hepatic acute phase protein whose expression is induced dramatically by an acute inflammatory stimulus or IL-6 injection. There are 2 major acute phase isoforms of this protein (SAA1.1 and SAA2.1) encoded by two separate but neighboring genes that are divergently transcribed and have similar but not identical amino acid sequences. Both proteins are predominantly associated with HDL or HDL like particles in the plasma [54]. Recent studies have focused on the capacity of the acute phase SAA isoforms to promote cholesterol efflux. SAA promotes efflux via both ABCA1 dependent and independent mechanism [55]. SAA2.1 has the capacity to promote cholesterol efflux to HDL, while SAA 1.1 is relatively inactive [56]. ABC transporters appear to be involved though precisely how this occurs is not clear. Most of the activity of SAA2.1 appears to be attributable to modulation of intracellular cholesterol homeostasis, favoring free cholesterol which obviously increases the potential for increased egress from the cell. This occurs by inhibition of acyl-CoA acyl transferase (ACAT), the intracellular enzyme responsible for cholesterol esterification, and activation of cholesterol ester hydrolase (CEH), the cellular enzyme responsible for hydrolysis of cholesteryl esters [57]. Different domains of SAA2.1 are responsible for each of these actions. A peptide corresponding to residues 1-20 is responsible for the inhibition of ACAT, while a peptide corresponding to residues 74-103, the C terminal amino acids, is responsible for the activation of the hydrolase. The intervening peptides 21 -50 and 51-80 are inactive. A peptide corresponding to residues 1-20 of SAA 1.1 is inactive. This peptide differs from that of SAA 2.1 by 2 amino acids at positions six and seven, IG versus VH.

Small peptides

Most, if not all, of the above discussed peptides are amphipathic helices. Some surprisingly small peptides have been shown to have bioactivity. These are tetra peptides and are non-helical. Two tetrapeptides, KRES and FREL, are both active in the monocyte chemotactic assay, reduce LDL lipid peroxides, and increase HDL associated PON activity in apoE-/- mice [58]. KERS, an isomer of KRES, has neither of these activities.

In vivo properties of apolipoprotein derived peptides as anti-inflammatory agents

The basic properties of apolipoprotein derived peptides have been reviewed here and in other recent publications [15]. Most research has focused on the mimetic peptides derived from the properties of apoA-I helices and those peptides' effects on atherosclerosis, now widely recognized as a chronic inflammation. As described above this involves effects on macrophage cholesterol efflux, remodeling of HDL, and on modification of pro-inflammatory properties of LDL. But there is evidence that these mimetic peptides have anti-inflammatory properties that are not concerned with cholesterol/lipoprotein homeostasis. The effectiveness of 4F in a variety of other chronic diseases in which inflammation plays a significant role has been recently reviewed [59]. These entities include arthritis, renal disease, brain vessel integrity and obesity.

Two examples of the effectiveness of the peptides in acute inflammation have recently been published. In one case, Van Lenten and colleagues studied influenza virus infection of isolated type II pneumocytes [50]. Viral infection of these cells produced increased quantities of cytokines, particularly IFN α/β and IL-6, some of which may be activated by cleavage by caspases that are activated by viral infection. Also there was an increased production and cellular release of oxidized phospholipid. Most of these responses to virus infection were significantly attenuated by D4F treatment. Perhaps the attenuation of oxidized phospholipid accumulation by 4F is critical and compatible with the underlying properties of D4F.

The second example is seen in the partial protection of rats from acute sepsis induced by the cecal ligation and puncture injury [61]. Intraperitoneal injection of 10 mg/kg L4F reduces IL-6 production, reverses pathologic reductions in effective circulatory volume and cardiac output, and improves viability of the animal.

In vivo properties of apolipoprotein derived peptides as anti-atherogenic agents

Most of the remaining studies of the mimetic peptides in vivo have focused on atherosclerosis and atherosclerosis related pathology. The finding that D4F could induce a dramatic decrease in early atherosclerosis in mice created a good deal of interest in this field [62]. Since then several other studies have been conducted (Table 2). In most studies the apoE $^{-/-}$ mouse model has been employed. But 4F on its own is only effective on the early foam cell lesions, not on established lesions, unless the peptide is used along with statin treatment [25, 62-64]. The early lesions of high fat/cholesterol diet fed LDLR $^{-/-}$ mice also exhibited responses to D4F therapy [62]. There is a very good correlation between the atheroprotective effects of the 4F family of peptides, reactivity in the monocyte chemotactic assay, and their ability to bind oxidized lipids with high affinity. Specifically, 3F-2 and 5F, but not 3F-14 are active along with 4F in each of these properties [18, 65]. It is notable that the parent of the 4F peptide family, 2F or 18A, is without effect on atherosclerosis, despite its capacity to promote cholesterol efflux [34]. We have observed that the intraperitoneal injection of L4F into apoE $^{-/-}$ mice from age 10 to 14 weeks of age attenuates very early lesion development in the innominate artery and the ascending aortic arch, but the same treatment has no effect on more mature lesions i.e. in apoE $^{-/-}$ mice treated from the age of 20 to 28 weeks of age (manuscript in preparation). In contrast, treatment with the tandem 4F-Pro-4F peptide was without effect at either stage (14 or 28 weeks).

Several other apoprotein mimetic peptides have also been shown to influence atherosclerosis in animal models. The apoJ 113-122 peptide reduces aortic lesions (aortic root and aorta en

face) in 30 week apoE^{-/-} mice treated for 24 weeks i.e. this treatment was initiated at the age of five to six weeks [45]. The small tetrapeptides KRES and FREL also reduce aortic lesions, but not the inactive isomer KRES [58]. Atherosclerosis in aortic lesions of 14 week old apoE^{-/-} mice fed a high-fat diet containing cholate was reduced by two weeks of treatment with either SAA 2.1 1-20 or 74 -103 peptides in liposomes [57]. This reduction was further enhanced when both SAA2.1 peptides were administered simultaneously. Finally, the apoE related peptide, AT1-5261, when administered either as free peptide or as a complex with POPC was effective in reducing atherosclerosis at the aortic root or in the aorta when administered for six weeks by intraperitoneal injection to either LDLR^{-/-} mice fed a high-fat diet or to apoE^{-/-} mice [53].

A few other models of vascular pathology have been studied. One of the consequences of hypercholesterolemia is a reduced capacity of blood vessels to relax. Only two weeks of treatment with 4F is sufficient to improve arterial vasorelaxation in LDLR^{-/-} mice on a high-fat diet [66, 67]. Ac-hE-18A-NH₂ has also been shown to improve vasorelaxation in Watanabe heritable hyperlipidemic rabbits [51]. The 5A peptide has also been shown to be active in vascular pathology. When administered as a complex with phospholipid, 5A decreases the expression of adhesion molecules in rabbit arteries [68]. The 4F peptide also reduced atherogenesis in the inferior vena cava transplanted into the carotid artery, but did not influence the endogenous arterial atherosclerosis [63].

In previous reviews, we discussed potential mechanisms by which these peptides afford atheroprotection. These include the remodeling of HDL to liberate free apoA-I that might promote reverse cholesterol transport; the sequestration of oxidized lipids that play an important role in early atherogenesis; and the promotion of reverse cholesterol transport. To this latter must be added an ABCA1 dependent reduction of cytokine production by macrophages, a STAT3-dependent phenomenon [69]. This is a potential role of ABCA1 interacting peptides which includes most of those discussed in this chapter. However, so far no definitive experiments have been done to establish that this mechanism of atheroprotection operates in vivo. Indeed none of these potential mechanisms has been established as the sole basis for the attenuation of atherogenesis by peptides, despite the excellent correlation between the established properties of the peptides in vitro and in culture experiments and their efficacy in vivo. It is clear from the ex vivo experiments that most peptides may operate through more than one of these mechanisms. Thus, 4F can remodel HDL, can sequester the characteristic pathogenic oxidized lipids, can promote reverse cholesterol transport, and its interaction with ABCA1 can activate the autophosphorylation of JAK2 and possibly Stat3 with resultant reduction in cytokine outputs. But which of these mechanisms is dominant in vivo remains to be established by further careful experimentation in animal models. It has been repeatedly observed that the peptides lead to a reduction in plasma lipid peroxides and an apparent increase in PON activity. These two parameters often appear to be coupled and may be at least in part mechanistically related. PON1 is an HDL associated enzyme that has the capacity to cleave oxidized phospholipid, mostly in the β -position, which is normally where the oxidized fatty acid is located. PON1 activity is dependent on its interaction with apoA-I, forming a high affinity complex between these two molecules [70, 71]. It remains to be established whether apoA-I related peptides also stabilize and activate PON1. And PON1 not only has the capacity to cleave oxidized phospholipid but can itself be inactivated by oxidized lipids [72]. Experiments with PON1 knockout mice have emphasized the role of this enzyme in atherogenesis.

Conclusions and prospects

It is clear that apolipoprotein derived peptides offer much promise as anti-inflammatory agents in a number of settings, and in atherosclerosis in particular. Initial studies treating a small number of cardiovascular patients with a single oral dose of D-4F have examined pharmacokinetics of the peptide and demonstrated that the peptide is safe and reduces the inflammatory index of the HDL without changes in plasma lipids or lipoproteins [73]. Additional clinical trials are in progress. But a great deal of further work is required to fully capitalize on D4F or related potentially promising therapeutic compounds.

- Significantly more work on the pharmacodynamics and pharmacokinetics of these agents is required. Until quite recently, work on these peptides has been limited by the expense of peptide production, especially for oral administration, since peptides synthesized from L amino acids are unsuitable for therapy due to their susceptibility to proteolysis. The repeated intraperitoneal or intravenous therapy in patients with peptides for prolonged periods to attenuate atherogenesis is not a feasible option. Hence the development of the D amino acid peptides that can be administered orally. Recently at least one strategy has been described for the incorporation of L4F into the diet [25]. Others are under development. More information is needed about the pharmacokinetics of the peptide including the rate of absorption and clearance and tissue distribution after administration.
- Most of the work so far published has not provided a detailed dose response and time course of effects on the different stages of atherosclerosis, which would be most helpful for clinical use of peptide therapy.
- Further investigation into whether complexing the peptides with phospholipids increases the efficacy of the peptides in vivo is needed.
- The great virtue of statins is that among many other actions they regulate the hepatic expression of the LDL receptor. If one could increase the activity of the available hepatic LDL receptor this could give rise to an even more effective lipid lowering strategy without inordinate increase of statin dosage. With suitable routes of peptide delivery, the use of peptides containing the LDL receptor/heparin binding domain of apoE, may bring us closer to the goal [74]. With improved delivery technology, this bears very careful scrutiny as a potential adjunct therapy for dyslipidemia e.g. heterozygous familial hypercholesterolemia. No such studies have yet been reported, either with respect to dose, route of administration and in vivo efficacy in the long-term.
- The controversy that surrounds the interaction of monomeric peptides with HDL needs to be resolved by further work.
- As has been described above, 4F is highly effective in reducing early atherosclerotic lesion growth, but it is relatively ineffective on established lesions, which is a more clinically relevant target. Is this ineffectiveness simply a matter of achieving a high enough level of peptide in the plasma? A detailed dosimetry would easily resolve this issue. Or does this point to very different requirements for treating early predominantly foam cell lesions and the more complex established lesions? Assuming that the sequestration of oxidized lipids is a predominant mechanism of action of the 4F family of peptides, is one to conclude that these lipids are not
- There is clearly much room for further modification of either monomeric or tandem peptides to achieve yet more effective therapeutic agents.

Thus, despite the promise of peptide therapy for chronic inflammatory disease and atherosclerosis in particular, there is a great deal of future work that lies ahead for investigators in this field.

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Figure 1.
Helical wheel depiction of the 18A mimetic peptide. Hydrophobic residues are yellow, acidic residue are red and basic residues are blue.

Table 1

In vitro effects of apoprotein mimetic peptides

Name	Cholesterol Efflux	Stabilize ABCA1	Antioxidative in MCA	Lipid and Lipoprotein Effects	Reference
<i>Peptides of Human ApoA-I Helices</i>					
apoA-I helix 1 (44-65)	—	—			41
apoA-I helix 10 (220-241)	—	—			41
apoA-I helix 1/9 chimera	+	+			41
apoA-I helix 9/10 chimera	+	+			41
apoA-I helix 10/9 chimera	—	—			41
<i>Monomeric ApoA-I Mimetic Peptides</i>					
2F (Ac-18-NH ₂)	+	+	Weak	binds VLDL and displaces apoE and apoCs	10, 16, 26, 75
3F-1			+		16, 17
3F-2			+	binds HDL>VLDL>IDL/LDL binds oxidized PL with > affinity than apoA-I	17, 18, 23
3F-3			—		16, 17
3F-14			—	binds VLDL/IDL/LDL>HDL binds oxidized PL with $\approx$ affinity as apoA-I	16-18, 23
4F	+	+	+	reduces LDL oxidation binds oxidized PL with > affinity than apoA-I	16, 17, 23, 25, 26, 33, 62
5F			+	activates LCAT	16, 65
6F			+	activates LCAT	16
7F			weak		16
<i>Tandem ApoA-I Mimetic Peptides</i>					
37pA (18A-Pro-18A)	+	+		increases secretion of apoA-I and HDL from HepG2 cells binds VLDL and displaces apoE and apoCs detergent-like effects may account for increased cholesterol efflux	10, 24, 26, 37, 39, 75
5A (18A-Pro-18A with Ala substitutions)	+ (>37pA)			no detergent-like effects	38
37aA (18A-Ala-18A)	+				37
4F-IHS-4F	+ (>4F)			remodels HDL to >extent than 4F	33
4F-Ala-4F	+ (>4F)			remodels HDL to >extent than 4F increases LDL oxidation	33

Name	Cholesterol Efflux	Stabilize ABCA1	Antioxidative in MCA	Lipid and Lipoprotein Effects	Reference
4F-Pro-4F	+ (>4F)			binds HDL	33
<i>Peptides mimicking other apoproteins</i>					
[113-122]apoI				↑PON ↓ LOOH	45
apoE Y(141-155)2				binds LDL receptor	48
apoE Y(141-p-155)2				does not bind LDL receptor	48
apoE AcY(141-155)2				binds LDL receptor binds VLDL, IDL/LDL, HDL	48
Ac-hE-18A-NH ₂ [(141-150)-18A]	+ (ABCA1 independent)			binds VLDL and LDL ↑ VLDL and LDL uptake by cells displaces apoE from VLDL ↓ LPS induced inflammatory response of EC	49, 50, 76
ATL-5261	+				53
mSAA1.1(1-20)	+				56, 57
mSAA2.1(1-20)	+ (↓ ACAT activity)				56, 57
mSAA2.1(74-103)	+ (↑ CEH activity)				56, 57
hSAA1.1/2.1(1-23)	+ (↓ ACAT activity)				56, 57
<i>Generically amphipathic Peptides</i>					
KRES			+		58
KERS			—		58
FREL			+	↓ LOOH ↑ PON activity	58

+ = positive effect; — = no effect; blank cells = no information

Table 2
In vivo effects of apoprotein mimetic peptides

Name	Lipid and Lipoprotein Effects	Atherosclerosis and Vascular Effects	Reference
<i>Monomeric ApoA-I Mimetic Peptides</i>			
2F (Ac-18-NH ₂)	elutes with HDL	No effect	34, 77
3F-2	associates with HDL	↓ aortic root lesion in 10 wk old apoE ^{-/-} mice (6 wk treatment)	18
3F-14	associates with apoB containing lipoproteins	No effect on aortic root lesions in 10 wk old apoE ^{-/-} mice (6 wk treatment)	18
4F	↓ plasma cholesterol (oral delivery, not IP)	No effect on aortic root lesion in 20 wk old apoE ^{-/-} mice fed HFD (4 wk treatment)	63
	No effect	↓ aortic root in LDLR ^{-/-} mice (6 wk treatment in liposomes)	62
	No effect	↓ aortic root in 9 wk old apoE ^{-/-} mice (4 wk treatment in water)	62
	↓ plasma cholesterol ↑ plasma triglyceride ↓ SAA	↓ en face aortic lesions in HDF fed rabbits (4 wk treatment SC)	78
	↑ HDL cholesterol and PON activity	↓ lesions in 21 wk old apoE ^{-/-} mice treated with statin (17 wk treatment) ↓ lesions in 1 yr old apoE ^{-/-} mice treated with statin (6 month treatment)	64
		↓ aortic root and en face aortic lesions in 15.5 month old apoE ^{-/-} mice treated with statin (6 month treatment)	25
	elutes with HDL and preβ-HDL (LDLR ^{-/-} mice) ↑ PON activity (monkey) ↓ LOOH in human HDL		34
	↑ reverse cholesterol transport ↓ LOOH in VLDL/LDL/HDL (<24 hr treatment) ↑ LOOH in preβ-HDL		31
	does not elute with HDL no effect on PON no effect on plasma lipids ↑ antibodies to oxidized phospholipids	No effect on lesions in 28 wk old apoE ^{-/-} mice (8 wk treatment IP) ↓ aortic arch and innominate artery lesions in 14 wk old apoE ^{-/-} mice (4 week treatment IP)	35, Wool et al. unpublished
	No effect	↑ vasodilation in LDLR ^{-/-} on HFD (2-6 wk treatment)	66, 67
5F	binds HDL ≫ VLDL decreases HDL in cholate diet fed mice	↓ aortic lesion in wild type mice on cholate diet (16 wk treatment)	65
<i>Tandem apoA-I Mimetic Peptides</i>			
37pA (18A-Pro-18A)	elutes with HDL		77
5A (18A-Pro-18A with Ala substitutions in 2 nd helix)		↓ adhesion molecule expression with 5A/PL complexes (rabbits)	68
4F-Ala-4F	no effect with 1 wk treatment		35

Name	Lipid and Lipoprotein Effects	Atherosclerosis and Vascular Effects	Reference
4F-Pro-4F	↑ IDL/LDL levels no effect on PON ↓ SAA (short term treatment) ↑ antibodies to oxidized phospholipids	no effect on lesions in 14 wk old apoE ^{-/-} mice (4 week treatment) or 28 wk old apoE ^{-/-} mice (8 wk treatment)	35, Wool et al unpublished
<i>Peptides mimicking other apoproteins</i>			
[113-122]apoI	elutes in late HDL ↑ HDL ↓ total cholesterol ↑ PON	↓ aortic lesions in 30 wk old apoE ^{-/-} mice (24 wk treatment)	45
apoE AcY(141-155)2	-rapid ↓ VLDL/IDL		48
Ac-hE-18A-NH ₂ [(141-150)-18A]	↓ VLDL and LDL in mice and WHHL rabbits ↑ PON (WHHL rabbits) ↓ LOOH plasma levels (WHHL rabbits)	improves vasorelaxation (WHHL rabbits)	50, 51, 78
ATI-5261	no effect	↓ aortic lesions in LDLR ^{-/-} mice fed HFD for 13 weeks (6 wk treatment) ↓ aortic and aortic sinus lesions in apoE ^{-/-} mice fed HFD 18 wks (switched to chow diet and treated for 6 wks)	53
mSAA2.1(1-20)	no effect	↓ aortic lesions in 14 wk old apoE ^{-/-} mice fed HFD with cholate (2 wk treatment)	57
mSAA2.1(74-103)	no effect	↓ aortic lesions in 14 wk old apoE ^{-/-} mice fed HFD with cholate (2 wk treatment) additional therapeutic effect when co-administered with mSAA2.1 (1-20)	57
<i>Generically amphipathic Peptides</i>			
KRES	elutes with HDL ↑ HDL ↑ PON activity ↓ LOOH in LDL and HDL	- ↓ aortic lesions in 18 wk old apoE ^{-/-} mice (12 wk treatment)	58
KERS	does not bind HDL no effect on HDL levels no effect on PON	- no effect	58
FREL	elutes with HDL ↑ HDL ↑ PON activity ↓ LOOH in LDL and HDL	- ↓ aortic lesions in 18 wk old apoE ^{-/-} mice (12 wk treatment)	58