An Oral ApoJ Peptide Renders HDL Antiinflammatory in Mice and Monkeys and Dramatically Reduces Atherosclerosis in Apolipoprotein E–Null Mice


Objective—To determine the properties of a peptide synthesized from D-amino acids corresponding to residues 113 to 122 in apolipoprotein (apo) J.

Methods and Results—In contrast to D-4F, D-[113–122]apoJ showed minimal self-association and helicity in the absence of lipids. D-4F increased the concentration of apoA-I with pre-β mobility in apoE-null mice whereas D-[113–122]apoJ did not. After an oral dose D-[113–122]apoJ more slowly associated with lipoproteins and was cleared from plasma much more slowly than D-4F. D-[113–122]apoJ significantly improved the ability of plasma to promote cholesterol efflux and improved high-density lipoprotein (HDL) inflammatory properties for up to 48 hours after a single oral dose in apoE-null mice, whereas scrambled D-[113–122]apoJ did not. Oral administration of 125 μg/mouse/d of D-[113–122]apoJ reduced atherosclerosis in apoE-null mice (70.2% reduction in aortic root sinus lesion area, \(P = 4.3 \times 10^{-13} \), 70.5% reduction by en face analysis, \(P = 1.5 \times 10^{-6} \)). In monkeys, oral D-[113–122]apoJ rapidly reduced lipoprotein lipid hydroperoxides (LOOH) and improved HDL inflammatory properties. Adding 250 ng/mL of D-[113–122]apoJ (but not scrambled D-[113–122]apoJ) to plasma in vitro reduced LOOH and increased paraoxonase activity.


Key Words: atherosclerosis ■ apolipoprotein J ■ high-density lipoproteins ■ lipoproteins ■ inflammation

Recently there has been great interest in the use of apolipoprotein (apo)A-I and apoA-I mimetic peptides as potential therapeutic agents for atherosclerosis.1–7 The literature contains reports of only 2 small (18 residue) peptides with efficacy in mouse models of atherosclerosis, 4F and 5F. Both are examples of class A amphipathic helical peptides, and only 4F synthesized from D-amino acids (D-4F) has been reported to be active after oral administration.4–9 The ability of these peptides to inhibit atherosclerosis in mouse models has been closely correlated with the ability of these peptides to inhibit LDL-induced monocyte chemotactic activity (MCA) in a human artery wall coculture.9 While the apoA-I mimetic peptides have many properties in common with apoA-I, there are some important differences. Human apoA-I binds inflammatory lipids in such a way that the apoA-I–lipid complex can still activate cells to produce MCA (ie, if low-density lipoprotein [LDL] and human apoA-I are added together in a coincubation with human artery wall cells, the LDL is still able to stimulate the cells to produce MCA).10 However, if apoA-I is added to the artery wall cells and removed before addition of LDL or if apoA-I is incubated with LDL and then separated from the LDL before the addition of the LDL to the cells (a preincubation), the LDL cannot induce the cells to produce MCA.10 In contrast, after D-4F binds these inflammatory lipids, the peptide–lipid complex will not stimulate the cells to produce MCA (ie, adding D-4F and LDL together in a coincubation prevents the LDL from causing the cells to produce MCA).9,11

ApoJ also bound these inflammatory lipids so that they were inactive in a coincubation (ie, LDL added to human artery wall cells in the presence of apoJ could not induce MCA).12 We hypothesized that short amphipathic helical sequences in apoJ could mimic the action of apoJ. Using the LOCATE program13 we identified 17 potential G* amphipathic helices in the mature apoJ protein (Table). We synthesized seven of these sequences and tested them in our artery
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Sequences in the Mature Apolipoprotein J Protein Containing a Potential Class G* Amphipathic Helix as Determined With the LOCATE Program

<table>
<thead>
<tr>
<th>Sequence</th>
<th>#</th>
<th>Sequence</th>
<th># Amino Residues</th>
<th>Tested in Culture?</th>
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<tr>
<td>7–31</td>
<td>NELQEMNGOSKQYNKKEQIONAVNGV</td>
<td>25</td>
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<td>24–44</td>
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<td>45–76</td>
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<td>32</td>
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<td>77–92</td>
<td>PGVQNETMALWEECK</td>
<td>16</td>
<td>No</td>
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<td>93–108</td>
<td>PCLIKONMKEYAVCR</td>
<td>16</td>
<td>No</td>
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<td>113–122</td>
<td>LVRGOREEFL</td>
<td>10</td>
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<td></td>
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<td>132–143</td>
<td>MNGDRDSLENN</td>
<td>12</td>
<td>No</td>
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<td>146–156</td>
<td>QOTHRMLDVMOD</td>
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<td>158–171</td>
<td>FSRAASSIPEFQOQ</td>
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<td>224–238</td>
<td>PPELHMAQQAMDI</td>
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<td>249–259</td>
<td>PTEFIREQGDOOD</td>
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<td>275–289</td>
<td>RMKDOCCRECRSLV</td>
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<td>296–305</td>
<td>PQSKLRRDELDSLOAERLTKYNNELKSYQ</td>
<td>32</td>
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<td>336–357</td>
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<td>358–368</td>
<td>DOYLYWATTVA</td>
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<td>PSVVTWVKFDS</td>
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<td>406–426</td>
<td>PKFMETVAEKALQEQYKHHRE</td>
<td>21</td>
<td>Yes</td>
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</table>

wall cell culture model and all but 1 ([146–156]apoJ) was effective in decreasing LDL-induced MCA (Table). However, only 2 were as effective as the intact apoJ protein in decreasing LDL-induced MCA (sequences [113–122]apoJ and [336–357]apoJ); the other five were less active than the intact apoJ protein. These 2 sequences ([113–122]apoJ and [336–357]apoJ) were synthesized from all D-amino acids and tested in preliminary studies for their ability to inhibit atherosclerosis in apoE-null mice after oral administration. Only the sequence [113–122]apoJ inhibited lesion formation in vivo in these preliminary studies. Subsequent studies constitute the basis of this report and show that oral [113–122]apoJ renders high-density lipoprotein (HDL) antiinflammatory in both mice and monkeys and dramatically reduces atherosclerosis in apoE-null mice.

Methods

Materials

The peptide Ac-L-V-G-R-Q-L-E-E-F-L-NH₂ corresponding to amino acids 113 to 122 in apoJ (D-[113–122]apoJ), scrambled D-[113–122]apoJ (Ac-L-G-R-V-Q-L-E-E-F-L-NH₂), D-4F, and scrambled D-4F were synthesized with all D-amino acids using solid phase synthesis as previously described.4,6,9 14C-D-[113–122]apoJ was synthesized using solid-phase synthesis in which Gly of Fmoc-Gly and the acetylating agent acetic acid were ¹⁴C-labeled. D-4F was iodinated using the iodogen procedure (Pierce Chemical Co). All other materials were from previously cited sources.6,10

Animals

Female apoE-null mice on a C57BL/6J background were purchased from Jackson Laboratories and fed high-fiber Purina monkey chow, 8 biscuits twice daily. The UCLA Animal Research Committee approved all animal studies.

Lipoproteins, Cell Cultures, Monocyte Chemotaxis, and Lesion Scoring

Lipoproteins, cell cultures, and monocytes were prepared and mono-ocyte chemotaxis assays were performed as described.6,14 Plasma samples were fractionated by a gel permeation fast protein liquid (FPLC) system as previously described.6 Aortic lesions were scored as previously described.5,15 ApoA-I in pre-β migrating particles was determined in 2-D gels using Western blots, a Typhoon scanner, and Image Quant software as previously described16 and expressed as the percent of total apoA-I in pre-β migrating particles.

Other Procedures

Lipoprotein cholesterol concentrations, paraoxonase activity, and lipoprotein lipid hydroperoxides were determined as previously described.6 Circular dichroism studies were performed as previously described.9,17,18 Cellular cholesterol efflux studies were performed as previously described.19 Statistical significance was determined using Model I ANOVA and significance defined as *P<0.05.

Results


There was a significant concentration-dependent CD change with D-4F but not with D-[113–122]apoJ in PBS indicating a tendency for self-association for D-4F but not for D-[113–122]apoJ (data not shown). D-4F showed significant helicity in PBS (42%), which was only modestly improved in the presence of lysophosphatidylcholine (LysPC), sodium decyl sulfate (SDS), and trifluoroethanol (TFE) (eg, in 1% SDS the percent helicity was 56%). In contrast, D-[113–122]apoJ had relatively poor helicity in PBS (13%), but there was nearly a 3-fold increase in helicity in the presence of LysPC, SDS, and TFE (eg, in 1% SDS the percent helicity was 31%).

D-[113–122]ApoJ in Human Artery Wall Cell Cultures

D-[113–122]apoJ potently inhibited LDL-induced MCA in vitro in a coincubation (data not shown) and thus acts like apoJ13 and D-4F14 and is different from apoA-I.10


Based on the counts in the plasma after an oral dose of ¹⁴C-[113–122]apoJ or ²¹D-[113–122]apoJ both peptides showed low absorption in apoE-null mice (Figure 1A and 1B).

As shown in Figure 1A after an oral dose, D-[113–122]apoJ slowly associated with lipoproteins and was cleared from plasma much more slowly than D-4F (Figure 1B) indicating significant differences in the kinetics of association and clearance between D-[113–122]apoJ and D-4F. Additionally it should be noted that [113–122]apoJ (in contrast to D-4F) largely remained in the fractions to the right of mature HDL in the FPLC chromatogram even after 36 hours (compare Figure 1A to Figure 1B). Similar results were obtained when ¹⁴C-[113–122]apoJ was added to mouse chow instead of administering it in water (data not shown).

We previously reported that D-4F causes increased formation of pre-β HDL.6 Figure 1C again demonstrates that oral D-4F significantly increases the amount of apoA-I in pre-β
migrating particles in apoE-null mice and also demonstrates that scrambled D-4F and D-[113–122]apoJ did not.

The ability of apoE-null mouse plasma to promote cholesterol efflux from macrophages via the ABCA1 pathway was poor compared with normal human HDL, but after oral administration of either D-[113–122]apoJ or D-4F apoE-null mouse plasma was as effective as normal human HDL (Figure 1D).

Administering D-[113–122]apoJ to apoE-null mice converted their HDL to antiinflammatory within 4 hours and the HDL remained significantly antiinflammatory for up to 48 hours after a single oral dose, whereas the same dose of scrambled D-[113–122]apoJ had no effect (Figure 1E).

Figure 1F and 1G demonstrate that administering D-[113–122]apoJ to apoE-null mice for 24 weeks dramatically reduced atherosclerosis.

The ability of apoE-null mouse plasma to promote cholesterol efflux from macrophages via the ABCA1 pathway was poor compared with normal human HDL, but after oral administration of either D-[113–122]apoJ or D-4F apoE-null mouse plasma was as effective as normal human HDL (Figure 1D).
The assay controls are as described in Figure 1E. The values shown are the Mean±SD. *
P<0.001.

Oral D-[113–122]apoJ in Monkeys

We previously reported that oral D-4F reduced lipoprotein lipid hydroperoxides and improved HDL-inflammatory properties in monkeys.20 D-[113–122]apoJ also reduced lipoprotein lipid hydroperoxides in monkeys (Figure 2A and 2B) and also rendered monkey HDL antiinflammatory 3 hours after oral administration (data not shown). Consistent with the plasma kinetics seen in mice (Figure 1A, 1B, and 1E), lipoprotein inflammatory properties remained significantly improved for up to 24 hours after a single oral dose of D-[113–122]apoJ in monkeys (Figure 2C and 2D). After another wash out period of more than 1 week from the experiments described in Figure 2C and 2D the monkeys were bled (time zero) and then given by gastric gavage 20 mg of D-[113–122]apoJ and bled 6 hours later and paraoxonase activity was determined (Figure 3C) and increased paraoxonase activity (Figure 3D). Addition of ng/mL of D-[113–122]apoJ to Plasma In Vitro Decreases Lipid Hydroperoxides and Increases Paraoxonase Activity

We previously reported that addition of D-4F to human plasma in vitro at a concentration of 250 ng/mL reduced lipid hydroperoxides and increased paraoxonase activity.21 As shown in Figure 3A, apoE-null mouse plasma has very little HDL-cholesterol compared with the cholesterol in the fractions containing apoB and the distribution of lipoproteins was not changed with the addition of D-[113–122]apoJ versus scrambled D-[113–122]apoJ. Addition of 250 ng/mL of D-[113–122]apoJ (but not scrambled D-[113–122]apoJ) to apoE-null mouse plasma in vitro significantly reduced the lipid hydroperoxide content of LDL (Figure 3B) and HDL (Figure 3C) and increased paraoxonase activity (Figure 3D).

Discussion

In vivo, oral D-4F causes the movement of apoA-I into particles with pre-β mobility in apoE-null mice whereas D-[113–122]apoJ does not (Figure 1C). The ability of D-4F to form pre-β HDL may be attributable to the ability of D-4F to promote the separation of cholesterol from phospholipids.18 It is possible that this property of D-4F may accelerate the normal process whereby apoA-I dissociates from alpha mi-
Oral D-[113–122]apoJ dramatically reduced atherosclerosis in apoE-null mice (Figure 1F and 1G) and was associated with a significant increase in HDL-cholesterol levels (26.4±4.0 versus 32.4±1.1 mg/dL) and an increase in HDL paraoxonase activity. The magnitude of the reduction in atherosclerosis in the apoE-null mice approaches that reported4 for D-4F. However, D-4F has also been shown to dramatically synergize with statins and cause lesion regression in old apoE-null mice.23 Future studies will be required to determine whether this is also the case for D-[113–122]apoJ.

We have observed in 2 different monkey colonies (Figure 2 for a colony at UCLA and data not shown for a colony in Michigan) that Cynomolgus monkeys have proinflammatory HDL (ie, their HDL fails to inhibit LDL-induced MCA). This proinflammatory HDL in animals with very little LDL is probably a positive evolutionary selection factor because it would presumably enhance protection from infection via the innate immune system while not contributing to a negative selection factor (atherosclerosis) in the absence of elevated levels of apoB containing lipoproteins. Similar to D-4F,20 D-[113–122]apoJ rapidly reduced lipoprotein lipid hydroperoxide levels in Cynomolgus monkeys (Figure 2A and 2B) and improved lipoprotein inflammatory properties for up to 24 hours after a single oral dose (Figure 2C and 2D). The rapid onset of action in both apoE-null mice (Figure 1E) and monkeys (Figure 2A and 2B), coupled with the prolonged effects after a single oral dose in both species (Figures 1E, 2C, and 2D) compare favorably with reports4,6,20 on D-4F (and data not shown). The sustained effect of D-[113–122]apoJ after a single oral dose would be consistent with the prolonged residence-time in plasma (Figure 1A).

A common mechanism for the beneficial effects of both D-4F and D-[113–122]apoJ may relate to the ability of both peptides to bind and sequester oxidized lipids and activate antioxidant enzymes such as paraoxonase21 (Figure 3B through 3D). Bielicki and Forte reported that lipid hydroper-
oxides inhibit plasma lecithin:cholesterol acyltransferase activity (LCAT) and Forte and colleagues demonstrated altered activities of LCAT, paraoxonase, and platelet-activating factor acetylhydrolase in atherosclerosis-susceptible mice that correlated with plasma levels of oxidized lipids. One common mechanism for D-4F and D-[113–122]apoJ might be the binding and sequestration of oxidized lipids that inhibit a series of antioxidant enzymes. With these oxidized lipids effectively sequestered (and presumably removed from the circulation), the activity of these antioxidant enzymes might be released from inhibition leading to further destruction of the oxidized lipids and the start of a positive feedback loop that amplifies the destruction of these proinflammatory lipids and results in an antiinflammatory environment.

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References

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