A new synthetic class A amphipathic peptide analogue protects mice from diet-induced atherosclerosis

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Abstract **Several synthetic class A peptide analogues have been shown to mimic many of the properties of human apo A-I in vitro. A new peptide [acetyl-(AspTrpLeuLysAlaPheTyrAspLysValPheGluLysPheLysGluPhePhe)-NH_2; 5F], with increased amphipathicity, was administered by intraperitoneal injection, 20 μg/day for 16 weeks, to C57BL/6J mice fed an atherogenic diet. Mouse apo A-I (MoA-I) (50 μg/day) or phosphate-buffered saline (PBS) injections were given to other mice as controls. Total plasma cholesterol levels and lipoprotein profiles were not significantly different between the treated and control groups, except that the mice receiving 5F or MoA-I had lower high density lipoprotein (HDL) cholesterol when calculated as a percentage of total cholesterol. No toxicity or production of antibodies to the injected materials was observed. When HDL was isolated from high fat diet-administered mice injected with 5F and presented to human artery wall cells in vitro together with human low density lipoprotein (LDL), there were substantially fewer lipid hydroperoxides formed and substantially less LDL-induced monocyte chemotactic activity than with HDL from PBS-injected animals. Injection of human apo A-I produced effects similar to 5F on lipid peroxide formation and LDL-induced monocyte chemotactic activity, but injection of MoA-I was significantly less effective in reducing lipid hydroperoxide formation or lowering LDL-induced monocyte chemotactic activity. Mice receiving peptide 5F had significantly less aortic atherosclerotic lesion area compared with mice receiving PBS, whereas lesion area in mice receiving MoA-I was similar to that of the PBS-injected animals. This is the first in vivo demonstration that a model class A amphipathic helical peptide has antiatherosclerotic properties.—We conclude that 5F inhibits lesion formation in high fat diet-administered mice by a mechanism that does not involve changes in the lipoprotein profile, and may have potential in the prevention and treatment of atherosclerosis.**—Garber, D. W., G. Datta, M. Chaddha, M. N. Palgunachari, S. Y. Hama, M. Navab, A. M. Fogelman, J. P. Segrest, and G. M. Anantharamaiah. **A new synthetic class A amphipathic peptide analogue protects mice from diet-induced atherosclerosis. *J. Lipid Res.* 2001.** 42: **545–552.**

Supplementary key words cholesterol • lipoproteins • metabolism • aorta

Epidemiological studies show an inverse correlation of high density lipoprotein (HDL) and apolipoprotein (apo) A-I levels with the occurrence of atherosclerotic events (1). Injection of HDL into rabbits fed an atherogenic diet has been shown to inhibit atherosclerotic lesion formation (2).

Human apo A-I has been a subject of intense study because of its antiatherogenic properties. Exchangeable apolipoproteins, including apo A-I, possess lipid-associating domains (3, 4). Apo A-I has been postulated to possess eight tandem repeating 22mer sequences, most of which have the potential to form class A amphipathic helical structures (3, 5). Characteristics of the class A amphipathic helix include the presence of positively charged residues at the polar-nonpolar interface and negatively charged residues at the center of the polar face (4, 5).

Peptide analogues that mimic many of the properties of human apo A-I have been synthesized by a number of research groups, including our own. Similar to human apo A-I, many of these peptides strongly associate with phospholipids to form complexes (6, 7), promote cholesterol efflux from cholesterol-enriched cells (8, 9), activate the plasma enzyme lecithin:cholesterol acyltransferase (10), interact with lipoproteins (11), and remove "seeding molecules" such as metabolites of linoleic acid (hydroperoxyoctadecadienoic acid) and arachidonic acid (hydroperoxyeicosatetraenoic acid) from low density lipoproteins (LDL) (12, 13). We and others have extensively studied an

Abbreviations: 5F, acetyl-(AspTrpLeuLysAlaPheTyrAspLysValPheGluLysPheLysGluPhePhe)-NH_2; apo, apolipoprotein; BSA, bovine serum albumin; CLiP, column cholesterol lipoprotein profile; DMPC, dimyristoylphosphatidylcholine; EDTA, ethylenediaminetetraacetic acid; ELISA, enzyme-linked immunosorbant assay; HDL, high density lipoprotein; IDL, intermediate density lipoprotein; LDL, low density lipoprotein; LPDS, lipoprotein-deficient serum; MoA-I, mouse apo A-I; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; SDS, sodium dodecyl sulfate; TCA, trichloroacetic acid; VLDL, very low density lipoprotein.

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18-residue peptide, 18A (14, 15). The N- and C-terminals of 18A are unprotected; blocking the termini (by acetylation of the N-terminal and amidation of the C-terminal) increased both its lipid affinity and many of its apo A-I-mimicking properties (16). This blocked peptide, Ac-18A-NH_2, has two phenylalanine (Phe) residues, both on the hydrophobic face, and is referred to as 2F.

Animal experiments have shown that human apo A-I was able to inhibit atherosclerosis (2, 17–20) and that human apo A-I and the 18A peptide were able to inhibit lipopolysaccharide-induced mortality in mice (15). The present study was designed to test the hypothesis that a new class A peptide analogue, acetyl-(AspTrpLeuLysAlaPheTyrAspLysValPheGluLysPheLysGluPhePhe)-NH_2 (5F), can inhibit lesion development in atherosclerosis-susceptible mice. For this purpose, we used the C57BL/6J mouse strain, which on an atherogenic diet develops fatty streak lesions in the aortic sinus (21). The new peptide, 5F, was blocked, in that the amino terminal residue was acetylated and the carboxyl terminal residue was amidated. Increasing the number of Phe residues on the hydrophobic face of 18A would theoretically increase lipid affinity as determined by the computation described by Palgunachari et al. (22). A systematic substitution of residues in the nonpolar face of 18A with Phe could yield several peptides with higher lipid affinities. Peptide analogues of 2F with additional 2, 3, and 4 Phe residues had theoretical lipid affinity (λ) values of 13, 14, and 15 units, respectively. However, the λ values jumped four units if the additional Phe were increased from 4 to 5 (to 19 λ units). Increasing to 6 or 7 Phe produced a less dramatic increase (to 20 and 21 λ units, respectively). Therefore, we chose three additional Phe (for a total of 5 Phe; hence, the peptide's designation as 5F). The new peptide analogue 5F was compared with mouse apo A-I (MoA-I) for efficacy in inhibiting diet-induced atherosclerosis in these mice using peptide dosages based on the study by Levine et al. (15).

MATERIALS AND METHODS

Peptides

Peptide 5F was synthesized by solid-phase peptide synthesis (6). The purity of the synthetic peptide was established by analytical high performance liquid chromatography (HPLC) and ion-spray mass spectrometry. The peptide was dialyzed against distilled water and lyophilized before using.

MoA-I was isolated from the plasma of C57BL/6J mice [ethylenediaminetetraacetic acid (EDTA) plasma was purchased from Harlan Bioproducts for Science, Indianapolis, IN]. MoA-I was isolated using a combination of size-exclusion and reversed-phase column chromatography. Briefly, plasma density was adjusted to 1.21 g/ml by addition of KBr, and centrifuged at 50,000 rpm for 24 h at 4°C (Ti70 rotor; Beckman, Fullerton, CA). The top fraction was collected, dialyzed against water to remove KBr, lyophilized, and delipidated. The pellet was dissolved in Gn-DTT-Tris solution (3 M guanidine HCl, 1 mM dithiothreitol, and 10 mM Tris, pH 8.0), then dialyzed against the same solution using 12,000 MW-cutoff dialysis tubing in order to remove much of the apo A-II and C apolipoproteins from the sample. The sample was then dialyzed against water and lyophilized. The pellet was dissolved in fresh Gn-DTT-Tris solution, and proteins were separated by size-exclusion column chromatography using an XK26/100 column (2.6 × 100 cm) packed with bulk-phase Superose 12 (Pharmacia Biotech, Piscataway, NJ) equilibrated with Gn-DTT-Tris solution. The flow rate was 0.5 ml/min, and 2.5-ml fractions were collected. Fractions corresponding to the apo A-I peak were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and further purified by preparative C-18 reverse-phase HPLC (23). Identity of the protein was confirmed using a PE-Siox APT triple-quadrupole ion-spray mass spectrometry.

Mice

All experiments were performed using female C57BL/6J mice (Jackson Laboratory, Bar Harbor, ME). Mice were purchased at 6 weeks of age, and the diet studies were begun with mice at 8 weeks of age. Mice weighing 20–22 g were used in the turnover studies. The atherogenic diet used was the modified Thomas-Hartroft diet containing cholate (#TD88051; Teklad, Madison, WI) as described by Paigen et al. (21). All animal studies were prospectively reviewed and approved by the Institutional Animal Care and Use Committee of the University of Alabama at Birmingham.

Kinetic studies

The 5F peptide, MoA-I, and human apo A-I were labeled with ^{125}I by the method of Bilheimer, Eisenberg, and Levy (24). Mice were placed on the modified Thomas-Hartroft atherogenic diet for 4 weeks, at which time intraperitoneal injections of peptide or protein dissolved in 200-μl phosphate-buffered saline (PBS) were begun. Animals injected with MoA-I or human apo A-I received 50 μg/animal; those injected with 5F received 20 μg. Animals were not fasted for the kinetic studies, and blood samples were taken under xylazine-ketamine anesthesia from the retro-orbital sinus at 15, 30, and 45 min, and 1, 1.5, 2, 3, 4, 6, 8, 12, and 24 h following injection. Each animal provided three blood samples at different time points (all retro-orbital and alternating eyes), and at least three samples were collected from different animals at each time point. Samples were collected into heparinized capillary tubes, then placed in dry-EDTA-coated microcentrifuge tubes (Eppendorf); the plasma was separated by centrifugation. Duplicate 10-μl aliquots of each sample were taken for radioactivity determination using gamma counting (Cobra; Packard Instruments, Downers Grove, IL) for 10 min/sample. Total plasma volume was calculated as 4.2% body weight. Each sample was expressed as the percentage of injected counts/min in total plasma. Free ^{125}I was determined by trichloroacetic acid (TCA) precipitation (1 ml of 10% TCA per 10-μl plasma sample). Fitting to the kinetic model was done using all data points, rather than averages at each time point (PKAnalyst; MicroMath Scientific Software, Salt Lake City, UT).

Injection protocol and sample collection for lesion studies

Mice were acquired at 6 weeks of age and randomized into groups of 20, except that a negative control group of 10 received no treatments and was given standard rodent chow. At 8 weeks of age, the treatment groups were placed on a modified Thomas-Hartroft atherogenic diet (#TD88051; Teklad), and injections were begun. The diet was stored at 4°C and was used for no longer than 3 months after the manufacture date to minimize lipid oxidation. Animals were injected intraperitoneally daily for 16 weeks including weekends and holidays. Twenty mice in each group received daily injections of 200 μl PBS (as positive controls), 20 μg 5F in 200 μl PBS, or 50 μg

MoA-I in 200 μl PBS. Human apo A-I was not used in the atherosclerosis protection studies due to the high probability of antibody formation (20).

Lyophilized 5F peptide and MoA-I were prepared in vials, with each bottle containing sufficient peptide for one day's injection. The peptide or protein was lyophilized in PBS, and was dissolved in autoclaved Milli-Q water (Millipore Corp., Bedford, MA) on the day of injection. The injection volume for all groups was maintained at 200 μl/mouse/day.

Blood samples were taken under anesthesia by retro-orbital bleeding at study entry (prediet) and at the time of organ harvesting. At the end of the study (week 16; at the last bleeding), the heart and the liver were excised. The hearts were kept in 0.9% saline solution for about 1 h to eliminate blood and to permit the heart muscle to relax. They were then fixed in phosphate-buffered 4% formaldehyde for at least 1 week until sectioned. The livers were removed and weighed.

Histological evaluation

Histological evaluations were performed according to the method of Paigen et al. (21) with some modifications. Briefly, hearts were fixed for at least 1 week in the phosphate-buffered formaldehyde solution. After the lower two-thirds of the hearts were removed, the remaining tissue was frozen in freezing medium (OCT Tissue-Tek; Miles Laboratories Ltd, Elkhart, IN) and sectioned in a cryostat at −20°C. Alternate 20-μm sections were saved on slides and observed for the beginning of the aortic root. Sections were then collected for an additional 600 μm, or until the aortic cross section was rounded and the valve cusps were no longer evident. Slides were stained with Oil Red O, and counterstained with hematoxylin. Stained lesion cross-sectional areas were measured in consecutive slides 80 μm apart by image analysis (SigmaScan Pro; SPSS Science, Chicago, IL), and the average lesion area was determined for each aortic sinus over the 400-μm length (five slides) providing the greatest mean lesion area.

Cocultures, monocyte isolation, isolation of lipoproteins, determination of lipid hydroperoxides, and monocyte chemotactic activity

Cocultures of human artery wall cells, monocyte isolation, isolation of lipoproteins by ultracentrifugation from the plasma of normal human donors or from mouse plasma by fast protein liquid chromatography (FPLC), and determination of lipid hydroperoxides and monocyte chemotactic activity were performed as previously described (12, 13). All human subject participation was with informed consent approved by the University of California at Los Angeles Human Subjects Protection Committee. The protocol for testing mouse lipoproteins in the coculture was also as previously described (12, 13). Briefly, LDL and HDL were isolated by FPLC from plasma from mice fed the atherogenic diet and injected with vehicle (PBS), with peptide 5F at 20 μg/mouse, or with human or mouse apo A-I at 50 μg/mouse. The cocultures were treated with human LDL at 200 μg/ml LDL protein or mouse LDL at 200 μg/ml, or with 200 μg/ml human LDL + human HDL at 350 μg/ml of HDL protein or mouse HDL at 300 μg/ml. The cocultures were incubated with or without the above additions for 8 h at 37°C in the presence of 10% lipoprotein deficient serum (LPDS). The supernatants were collected and analyzed for Auerbach lipid hydroperoxide equivalents (25). The cocultures were then washed and incubated with fresh culture medium without serum or LPDS for an additional 8 h. The conditioned medium was collected and analyzed for monocyte chemotactic activity.

Chemical and analytical methods

Column cholesterol lipoprotein profiles (CLiP). Plasma CLiP were measured using our recently developed method (26). Briefly, 5–10 μl of plasma were analyzed using a single Superose 6 (Pharmacia Biotech) column. Immediately following the column, cholesterol reagent was introduced through a mixing tee, and the eluent-reagent mixture entered a postcolumn reaction coil. Cholesterol content of the eluent mixture was spectrophotometrically detected at 500 nm, and data points were collected into a computer. The resulting profiles were decomposed into component peaks and analyzed for relative area using PeakFit (SPSS Science); absolute cholesterol values for total cholesterol and each component peak were determined by comparison with a control sample of known values. In some cases, fractions were collected to determine distribution of radioactivity.

Antibody detection. To determine whether daily injections of peptides elicited any immune response in mice, indirect enzyme-linked immunosorbant assay (ELISA) titration (27) was carried out with plasma taken from mice at the time of organ collection (following 16 weeks of daily injection). Plates were coated with the injected peptides or MoA-I (10 μg/ml). Plates were incubated overnight. After thorough washing with borate-buffered saline (pH 8.2) containing 0.05% Tween 20, and blocking with buffer (0.1% gelatin and 0.1% BSA in borate buffer) for 1 h, 200 μl of the diluted mouse plasma (1:100 dilution) samples were serially diluted 1:1 with borate-buffered saline. Biotinylated goat antibody to mouse IgG (0.1 μg/ml) was then added to the wells and the plates were treated with Streptavidin-horse radish peroxidase for 1 h and developed with ABTS [2,2′-azino-bis-(3-ethylbenothiazine-6-sulfonic acid), diammonium salt] and peroxide as substrate. The plates were incubated overnight at room temperature after every addition of antigen/antibody, washed thoroughly with borate-buffered saline (pH 8.2) containing 0.05% Tween 20, and blocked with buffer (0.1% gelatin and 0.1% BSA in borate buffer) for 1 h before the next addition.

Statistical methods

Treatment groups were compared by two-tailed *t*-tests or one-way analysis of variance (where the data were normally distributed), or by one-way analysis of variance on ranks (SigmaStat; SPSS Science). Kinetics of peptide or protein turnover were analyzed by fitting to a first-order one-compartment kinetic model assuming nonequal input and output rates (PKAnalyst; MicroMath Scientific Software).

RESULTS

Physical/chemical properties of peptide 5F

Selected properties of peptide 5F are presented in **Table 1**. The solubility in PBS was low (0.1 mg/ml), and it was more helical when bound to lipid (dimyristoylphosphatidylcholine) than in aqueous solution, as determined by circular dichroism (28). It self-associated in the absence of lipid and did not displace apolipoproteins from mouse HDL under the conditions of the experiment. Detailed in vitro physical/chemical studies of this peptide will be reported separately.

Kinetic studies

The kinetics of the clearance of peptide 5F and human and mouse apo A-I from mouse plasma following intraperitoneal injection are summarized in **Table 2**. Human and mouse apo A-I had greatly prolonged clearance com-

TABLE 1. Selected physical/chemical properties of peptide 5F

Maximum solubility in PBS	0.1 mg/ml
% Helicity in PBS[a]	55%
% Helicity in lipid complex[a]	76%
Self-association in absence of lipid[b]	Yes
Displacement of apolipoproteins from HDL[c]	Did not displace apolipoproteins from HDL under the experimental conditions

[a] % Helicity was determined by circular dichroism (CD). CD spectra were recorded on an AVIV 62DS spectropolarimeter as described earlier (28). Briefly, spectra were obtained using a cell with a 0.1-cm path length, and measurements were taken every nanometer from 260 nm to 190 nm at 25°C. Owing to the poor solubility of 5F, peptide solutions in PBS (pH = 7.4) were used at a concentration of 11 μM. Peptide-DMPC complexes (1:20 mol:mol) were used to determine the effect of lipid binding on the helicity of this peptide.

[b] Self-association of the peptide in absence of lipid was determined by nondenaturing PAGE. 5F has a molecular mass of 2,429 Da. 3 μg of the peptide on a 4–20% native gel had a size similar to that of an HDL particle (like Ac-18A-NH_2, which also self associates, and unlike 18A, which does not) (16). However, 3 μg of the peptide on a 4–20% SDS gel had a molecular mass of approximately 3,000 Da.

[c] 5F was incubated with plasma from C56BL/6J mice on normal chow and on high-fat diet at a ratio (4 μg/ml plasma) near the maximal peptide blood level observed in kinetic studies (Table 2). Plasma density was adjusted to 1.21 g/ml and was centrifuged. The lipoprotein fraction was examined by SDS-PAGE. No change in the concentrations of apolipoproteins was observed.

pared with the 5F peptide. Human apo A-I and 5F had longer times-to-peak plasma levels than did mouse apo A-I, although human apo A-I reached higher peak levels than did the other two materials. Analysis of plasma samples by column chromatography demonstrated that peptide 5F and apo A-I (both human and mouse) associated with plasma lipoproteins, especially with particles in the HDL-sized region (**Fig. 1**). The HDL/VLDL ratio of peptide radioactivity 1.5 h following injection of 5F was 4.19 ± 0.58. Similar results were obtained 5 h following injection of 5F (6.44 ± 1.10). The injected peptide initially had less than 3% free ^{125}I as determined by TCA precipitation. However, after injection of peptide, free ^{125}I radioactivity in the plasma as a percentage of total eluted radioactivity was substantially greater, being 26.9 ± 9.4% at 1.5 h and 34.4 ±

TABLE 2. Summary of fitted data from kinetic experiments

Injected Material	$T_{1/2}$	Time to Maximum CPM	Max. % in Plasma	r^2
Human apo A-I (50 μg/mouse)	15.6	3.61	23.7	0.947
Mouse apo A-I (50 μg)	15.7	1.74	13.5	0.928
5F (20 μg)	6.22	2.36	14.29	0.895

Peptide or proteins were labeled with ^{125}I (24) and injected intraperitoneally to C57BL/6J mice that had been fed the atherogenic diet for 4 weeks. Blood samples were taken from the retro-orbital sinus at times indicated in Materials and Methods. Data shown represent results of fitting data to a first order one-compartment kinetic model assuming unequal input and output rates (PKAnalyst; MicroMath Scientific Software, Salt Lake City, UT). The dosage of 5F was used due to the limited solubility of this peptide and limitations in injection volume. $T_{1/2}$, half-time of clearance from plasma (hours); CPM, counts per minute; Max. % in plasma, percent of injected dose found in total plasma at peak levels; r^2, goodness-of-fit statistic of the kinetic model.

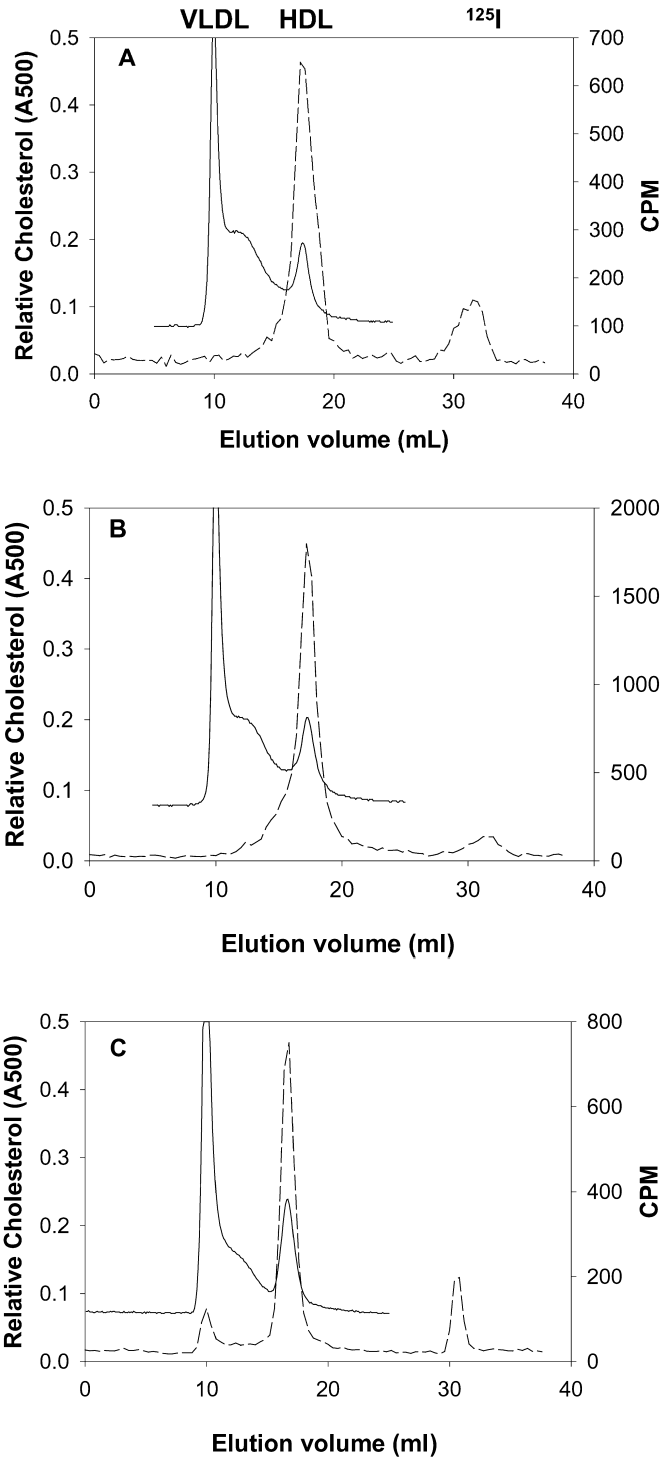

Fig. 1. Plasma distributions of peptide 5F or apo A-I following intraperitoneal injection. Human apo A-I, mouse apo A-I, and peptide 5F were labeled with ^{125}I and injected intraperitoneally into C57BL/6 mice that had been fed the atherogenic diet for at least 3 weeks. Samples were taken during the kinetic studies described in Table 1. Representative samples were analyzed by the CLiP method, and fractions were collected for determination of radioactivity. The elution volume was based on the column pump rate only; the volume contributed by the enzymatic reagent pump was neglected. Data shown are cholesterol (as absorbance at 500 nm in arbitrary units; solid lines) and radioactivity [in counts per min (CPM); dashed lines]. A: human apo A-I (1 h following injection); (B) mouse apo A-I (1 h), (C) 5F (1.5 h).

TABLE 3. Body and liver weights following treatment

Diet	Body Weight	Liver Weight	Liver Weight/ Body Weight
	g	*g*	%
Chow	23.38 ± 0.52	0.99 ± 0.02	4.24 ± 0.04
Atherogenic			
PBS (n = 14)	20.55 ± 0.32[a]	1.60 ± 0.04	7.84 ± 0.26
5F (n = 15)	21.60 ± 0.28	1.61 ± 0.04	7.46 ± 0.23
MoA-I (n = 14)	21.16 ± 0.34	1.72 ± 0.04	8.15 ± 0.23[a]

Data shown are mean ± SEM of weights taken at the time of organ harvesting (after 16 weeks of treatment). The chow-fed animals received no injections. The other mice were maintained on the atherogenic diet as described in Materials and Methods. The PBS group received intraperitoneal injections of 200 μl PBS daily. The 5F group received intraperitoneal injections of 20 μg 5F in 200 μl PBS daily and the MoA-I group received 50 μg MoA-I in 200 μl PBS daily.

[a] $P < 0.05$ versus 5F; two-tailed *t*-test.

4.8% at 5 h, reflecting the expected metabolism of lipoproteins and lipoprotein-associated peptide or proteins. The rate of increase in the radioactivity due to free iodine from 1.5 to 5 h was less than that from the time of injection to 1.5 h, possibly suggesting considerable initial degradation of the peptide in the peritoneal cavity.

Survival and gross morphology on the chow or atherogenic diets

Only three mice died from unexplained causes during the course of the prolonged peptide administration studies. Two of the animals had been receiving MoA-I, and one was receiving 5F peptide. At the time of organ collection, no gross morphological differences were observed between the groups. Livers were enlarged in all animals fed the atherogenic diet, but liver weights and liver weight as a percentage of body weight were not different between groups (**Table 3**). All animals on the atherogenic diet (including PBS-injected animals) had lower body weights than the chow-fed controls (Table 3).

Antigenicity

Blood samples taken at the conclusion of the 16-week injection period were tested for the presence of antibodies against the peptides. No antibodies were detected against peptide 5F or against MoA-I (data not shown). Cross-experiments, where the ELISA plates were coated with peptide or protein, which were not injected into that series of animals, produced results essentially identical to those in the direct determination of the presence of antibodies (data not shown).

Lipoprotein cholesterol characterization

Total and lipoprotein cholesterol values as determined by the CLiP method are presented in **Table 4**. Accuracy of total cholesterol values was confirmed by a manual cholesterol assay (Cholesterol 1000; Sigma, St. Louis, MO; data not shown). No significant differences in total or lipoprotein-fraction cholesterol levels were seen between the treatment groups. However, when lipoprotein fractions were expressed as a percentage of total cholesterol (Table 4), HDL cholesterol comprised a significantly lower percentage in the 5F and MoA-I groups compared with the PBS group.

Interaction of mouse lipoproteins with human artery wall cells

Figure 2 demonstrates that HDL from the mice in the present study that were fed the atherogenic diet and injected with PBS failed to inhibit the oxidation of human LDL (Fig. 2A) and failed to inhibit LDL-induced monocyte chemotactic activity (Fig. 2B) in human artery wall cocultures. In contrast, HDL from mice fed the atherogenic diet and injected daily with peptide 5F was as effective in inhibiting human LDL oxidation and preventing LDL-induced monocyte chemotactic activity in the cocultures as was normal human HDL. Figure 2 also shows that LDL taken from mice fed the atherogenic diet and injected daily with PBS was more readily oxidized and more readily induced monocyte chemotactic activity than LDL taken from mice fed the same diet but injected with 20 μg daily of peptide 5F. No cytotoxicity was noted in the artery wall cells treated with any of the lipoproteins (data not shown). Similar results were obtained in three separate experiments (data not shown). Injection of human apoA-I into mice fed the atherogenic diet produced results similar to those from 5F-injected mice, but results from injec-

TABLE 4. Total and lipoprotein cholesterol levels after 16 weeks of chow or atherogenic diet

Diet	VLDL	IDL + LDL	HDL	TC
Chow	11.66 ± 2.34 (16.61 ± 3.55%)	23.68 ± 3.51 (31.66 ± 3.61%)	7.30 ± 2.52 (51.73 ± 1.75%)	72.64 ± 5.58
Atherogenic				
PBS-injected	88.36 ± 5.48 (47.26 ± 1.37%)	75.82 ± 7.64 (39.83 ± 1.34%)	24.36 ± 2.19 (12.91 ± 0.68%)	188.54 ± 14.22
5F-injected	100.34 ± 15.72 (47.96 ± 3.26%)	83.37 ± 8.15 (42.80 ± 2.51%)	17.92 ± 2.91 (9.24 ± 1.18%)[a]	201.63 ± 25.21
MoA-I injected	100.08 ± 9.73 (48.23 ± 2.75%)	87.86 ± 8.34 (42.44 ± 2.46%)	19.50 ± 3.07 (9.34 ± 1.19%)[a]	207.45 ± 16.94

Data are expressed as mean mg/dl ± SEM and, in parentheses, as percent of total cholesterol (TC). The chow-fed animals received no injections. The other mice were maintained on the atherogenic diet as described in Materials and Methods. The PBS group received intraperitoneal injections of 200 μl PBS daily. The 5F group received intraperitoneal injections of 20 μg 5F in 200 μl PBS daily and the MoA-I group received 50-μg MoA-I in 200-μl PBS daily. Numbers of animals shown are as in Table 3.

[a] $P < 0.05$ or less compared with PBS by two-tailed *t*-test.

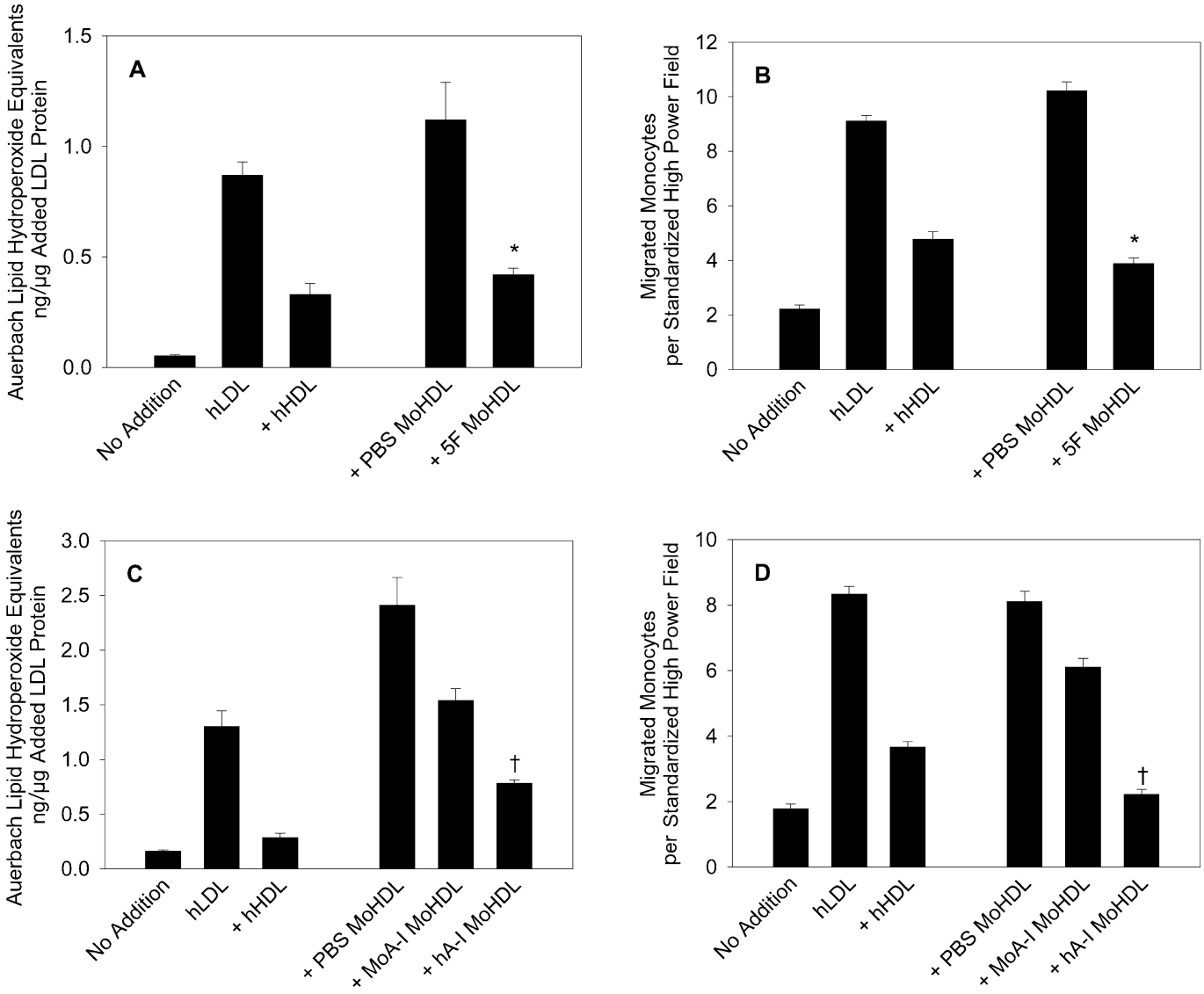

Fig. 2. Interaction of mouse lipoproteins with human artery wall cells. LDL and HDL were isolated by fast protein liquid chromatography from the plasma of mice fed the atherogenic diet and injected with vehicle (PBS), peptide 5F at 20 μg/mouse, human apo A-I (hA-I) at 50 μg/mouse, or mouse apo A-I (MoA-I) at 50 μg/mouse. Blood was collected 4 h later using heparin as the anticoagulant. The cocultures were treated without (No Addition) or with human LDL (hLDL) at 200 μg/ml LDL protein, or with 200 μg/ml human LDL + human HDL (hHDL) at 350 μg/ml of HDL protein or mouse HDL (MoHDL) at 300 μg/ml. The cocultures were incubated with the above additions for 8 h at 37°C in the presence of 10% LPDS. The supernatants were collected and analyzed for Auerbach lipid hydroperoxide equivalents (A and C: n = 4 observations for each condition). The cocultures were then washed and incubated with fresh culture medium without serum or LPDS for an additional 8 h. The conditioned medium was collected and analyzed for monocyte chemotactic activity (B and D: n = 9 observations for each condition). Data are expressed as mean ± SEM. * $P < 0.001$ comparing hLDL + PBS MoHDL with hLDL + 5F MoHDL. † $P < 0.001$ comparing hLDL + MoA-I MoHDL with hLDL + hA-I MoHDL.

tion of MoA-I were more similar to those from PBS-injected animals (Fig. 2C and D).

Lesion formation

Mean lesion cross-sectional areas are presented in **Fig. 3**. As expected, no lesions were observed in the group fed normal mouse chow (data not shown). However, high fat diet administration produced the previously reported changes in lipoprotein cholesterol levels (21) (Table 4), as well as lesion formation (Fig. 3). The 5F-injected animals had significantly lower mean lesion area than PBS-injected animals, whether analyzed by two-tailed *t*-test ($P < 0.002$) or by one-way analysis of variance on ranks ($P < 0.001$; determined due to the non-normal distribution of mean lesion areas). MoA-I injection produced no difference in lesion area compared with PBS injection, and lesion area was significantly greater than in 5F-injected animals, both by *t*-test ($P < 0.002$) and by one-way analysis of variance on ranks ($P < 0.001$).

It was confirmed in a separate experiment using only PBS and 5F injections with the same protocol (though with fewer animals) that 5F inhibited high fat diet-induced atherosclerotic lesion formation, again showing significantly less extensive lesions in the 5F-injected mice

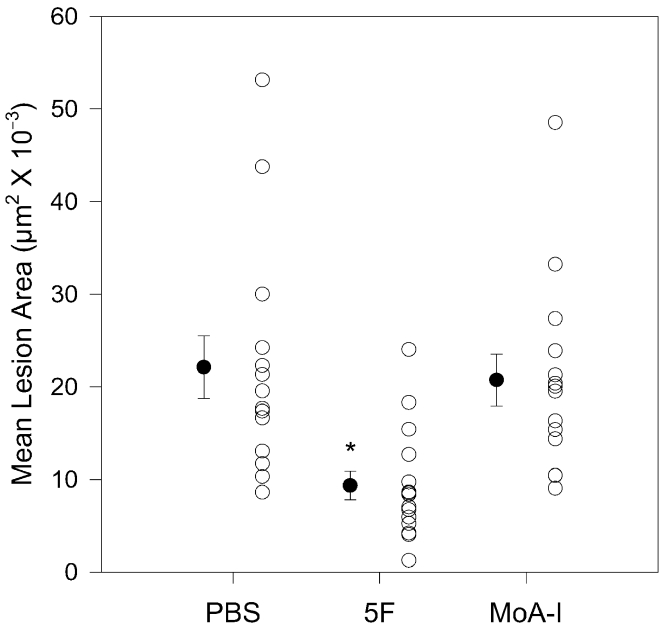

Fig. 3. Mean lesion cross-sectional areas. Data shown represent the mean lesion cross-sectional area for each animal (open circles) and the mean ± SEM of all animals in each group (closed circle with error bars). Abbreviations: PBS, mice fed the atherogenic diet and injected daily with 200 μl PBS; 5F, mice fed the atherogenic diet and injected daily with 20 μg of 5F in 200 μl PBS; MoA-I, mice fed the atherogenic diet and injected daily with 50 μg of mouse apo A-I in 200-μl PBS. * $P < 0.002$ as determined by two-tailed *t*-test. A significant difference was also shown using one-way analysis of variance on ranks ($P < 0.001$).

compared with controls (PBS: n = 7, mean lesion area = 35.7 ± 5.4 × 10^3 μm²; 5F: n = 5, mean lesion area = 20.1 ± 4.0 × 10^3 μm²; $P < 0.025$ by two-tailed *t*-test as the data were normally distributed).

DISCUSSION

This study demonstrates that a model class A amphipathic helical peptide has antiatherogenic properties in vivo, similar to human apo A-I. The mechanism by which the peptide is protective is not through direct modification of lipoprotein levels. Although the antiatherogenic mechanism is not completely understood, the peptide does exhibit striking antioxidative properties as determined by inhibition of LDL-mediated monocyte chemotaxis.

The studies presented here demonstrated that peptide 5F entered the plasma after interperitoneal injection and achieved plasma levels that were roughly comparable to MoA-I, but less than human apo A-I (Table 1 and Fig. 1). The plasma clearance half-time of 5F was shorter than either mouse or human apo A-I after peritoneal injection. After injection, the majority of 5F was found in the region of HDL (Fig. 1), despite the fact that the preponderance of circulating cholesterol was in the VLDL-, IDL-, and LDL-sized particles of plasma from mice on the atherogenic diet.

Plasma cholesterol levels and distributions were not significantly different among the injected groups on the atherogenic diet (Table 4). However, when the lipoprotein fractions were expressed as a percentage of total cholesterol (Table 4), HDL cholesterol comprised a significantly lower percentage in the 5F and MoA-I groups compared with the PBS group.

We recently reported (12, 13) that normal HDL inhibits three steps in the formation of mildly oxidized LDL. In those studies we demonstrated that treating human LDL in vitro with apo A-I or an apo A-I-mimetic peptide removed seeding molecules from the LDL that included oxidation byproducts of arachidonic and linoleic acid. These seeding molecules were required for cocultures of human artery wall cells to be able to oxidize LDL and for the LDL to induce the artery wall cells to produce monocyte chemotactic activity (12, 13). We also demonstrated that after injection of human apo A-I into mice or infusion into humans, the LDL isolated from the mice or human volunteers after injection/infusion of human apo A-I was resistant to oxidation by human artery wall cells and did not induce monocyte chemotactic activity in the artery wall cell cocultures (12, 13). In the present studies, HDL from mice that were fed the atherogenic diet and injected with PBS failed to inhibit the oxidation of human LDL (Fig. 2A) and failed to inhibit LDL-induced monocyte chemotactic activity (Fig. 2B) in the human artery wall cocultures. Unlike human apo A-I, injection of high fat diet-administered mice with MoA-I also failed to inhibit oxidation of human LDL and LDL-induced monocyte chemotaxis (Fig. 2C and D). In contrast, HDL from mice fed the same atherogenic diet but injected with peptide 5F was found to be as effective in inhibiting human LDL oxidation and preventing LDL-induced monocyte chemotactic activity in the cocultures as was normal human HDL (Fig. 2A and B). It is possible that 5F interacted with LDL in the circulation (either before or after associating with HDL) and removed seeding molecules necessary for LDL oxidation and LDL-induced monocyte chemotactic activity in a manner similar to that described in vitro for a related peptide, 18A-Pro-18A (12, 13).

The in vitro responses of human artery wall cells to HDL and LDL from mice fed the atherogenic diet and injected with peptide 5F were consistent with the protective action of 5F in vivo. Despite similar levels of total cholesterol, LDL cholesterol, IDL + VLDL cholesterol, and lower HDL cholesterol as a percentage of total cholesterol, the animals fed the atherogenic diet and injected with 5F had significantly lower lesion scores (Fig. 3). These results were somewhat analogous to those of Shah et al. (20), who found that apo $A\text{-}I_{Milano}$ prevented progression of atherosclerotic lesions in apo E-deficient mice, despite persistence of hypercholesterolemia.

MoA-I in this study was markedly ineffective in preventing formation of LDL hydroperoxides or inhibiting LDL-induced monocyte chemotaxis (Fig. 2C and D), suggesting that it differs from human apo A-I in important biophysical and physiological properties. It has been shown that MoA-I does not form as stable protein/lipid complexes as does human apo A-I (29). Mouse HDL has also been shown to be more easily denatured by guanidine hydro-

chloride than human HDL (29), suggesting that amphipathic helical peptides might displace MoA-I more easily from mouse HDL than human apo A-I from human HDL. The dosage of MoA-I injected was not sufficient to raise plasma HDL levels in these mice (Table 4). In any event, 5F peptide was significantly effective under these conditions and MoA-I was not.

The ELISA analysis of plasma at the conclusion of the injection protocol indicated that antibodies were not formed against the 5F peptide. This was not surprising, in that lipid-associating peptides have been shown not to produce antibodies, presumably because these peptides bind lipids in such a way as to prevent the exposure of epitopes necessary to elicit an immune response (30, 31).

A preliminary study by us suggested that transgenic mice expressing a class A amphipathic helical peptide (18A-Pro-18A) with theoretically less lipid affinity than the peptide used in this study may have been resistant to atherosclerosis (32). The current study suggests that peptide 5F likely has great potential for elucidating the mechanisms involved in atherogenesis and may also have therapeutic potential.

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