Lipoprotein inflammatory properties and serum amyloid A levels but not cholesterol levels predict lesion area in cholesterol-fed rabbits

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Abstract Rabbits on a 1% cholesterol diet received injections of vehicle with or without D-4F or L-4F. After 1 month, the percent of aorta with atherosclerotic lesions was 24 ± 15% (vehicle), 10 ± 6% (D-4F) ($P < 0.01$ vs. vehicle), and 13 ± 9% (L-4F) ($P < 0.05$ vs. vehicle). Inflammatory indexes for HDL and LDL were determined by measuring monocyte chemotactic activity after adding rabbit lipoproteins to human endothelial cells. HDL-inflammatory index (HII) and LDL-inflammatory index (LII), respectively, were 1.39 ± 0.24; 1.35 ± 0.29 (vehicle), 0.67 ± 0.26; 0.63 ± 0.38 (D-4F) ($P < 0.001$ vs. vehicle), and 0.67 ± 0.2; 0.68 ± 0.32 (L-4F) ($P < 0.01$ vs. vehicle). Serum amyloid A (SAA) levels were 95 ± 39, 8 ± 22, and 7 ± 19 μg/ml, respectively, for vehicle, D-4F, and L-4F ($P < 0.001$ vs. vehicle). There was no correlation between lesion area and total plasma or HDL-cholesterol levels. In contrast, there was a positive correlation with HII, LII, and SAA ($P = 0.002$, $P = 0.0026$, $P = 0.0079$, respectively). HII correlated closely with SAA levels ($r = 0.6616$; $r^2 = 0.4377$, $P < 0.0001$). Thus, HII, LII, and SAA are better predictors of lesion area than are total plasma or HDL-cholesterol levels in cholesterol-fed rabbits.—Van Lenten, B. J., A. C. Wagner, M. Navab, G. M. Anantharamaiah, S. Hama, S. T. Reddy, and A. M. Fogelman. **Lipoprotein inflammatory properties and serum amyloid A levels but not cholesterol levels predict lesion area in cholesterol-fed rabbits.** ***J. Lipid Res.*** **2007.** 48: **2344–2353.**

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Recent reports have suggested that the inflammatory properties of HDL may predict susceptibility to atherosclerosis in mice and humans better than HDL-cholesterol levels (1–7). Although lesion area can be measured in individual mice, lipoprotein inflammatory properties must be determined on pooled samples (four to five mice per pool) because of the amount of plasma required for these assays. The conclusion that lipoprotein inflammatory properties predicted atherosclerosis was inferred after treatment with apolipoprotein mimetic peptides, because there was a change in the same direction in both lipoprotein inflammatory properties and lesion area without a change in plasma cholesterol levels (1). This conclusion was not directly determined from a mathematical correlation of lipoprotein inflammatory properties and lesions in the same animal because of the inability to obtain sufficient plasma from mice for these assays. Additionally, to date, there has been no correlation between a well-established marker of inflammation such as serum amyloid A (SAA) and assays that measure the inflammatory properties of lipoproteins by their ability to induce endothelial cells to produce monocyte chemoattractant protein-1 (MCP-1) or to inhibit them from doing so. The rabbit is large enough to allow the measurement of lipoprotein inflammatory properties and lesion areas in the same animal. Therefore, to directly test the importance of lipoprotein cholesterol levels versus their inflammatory properties in an animal model of atherosclerosis and to compare these assays with a well-established marker of inflammation (SAA), we studied cholesterol-fed rabbits that were sham-treated or were treated with an apolipoprotein A-I (apoA-I) mimetic peptide (4F) that had previously been shown to have potent anti-atherosclerotic and vasoprotective properties in mouse models of atherosclerosis (8–15) and in a rat model of diabetes and vascular disease (16).

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Abbreviations: apoA-I, apolipoprotein A-I; FPLC, fast protein liquid chromatography; HII, HDL-inflammatory index; LII, LDL-inflammatory index; MCP-1, monocyte chemoattractant protein-1; SAA, serum amyloid A.

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MATERIALS AND METHODS

Peptides

The peptide Ac-D-W-F-K-A-F-Y-D-K-V-A-E-K-F-K-E-A-F-NH2 was synthesized from all D-amino acids (D-4F) or all L-amino acids (L-4F) as previously described (8). Additionally, a scrambled peptide with the same amino acids as in 4F but in a sequence (Ac-D-W-F-A-K-D-Y-F-K-K-A-F-V-E-E-F-A-K-NH2) that does not promote α helical formation was synthesized from L-amino acids as previously described (11) as a control peptide for some studies.

Rabbits

Forty-five female New Zealand White rabbits weighing 2.2–2.6 kg were purchased from Charles River Laboratories at 3 months of age and maintained on normal rabbit chow (Harlan Teklad; catalog # TD 0533). At 7 months of age, the rabbits weighed 3.83 ± 0.3 kg, and after an overnight fast, blood was collected in tubes containing heparin (2.6 USP units per milliliter of blood) for determination of plasma lipid levels, and they were started on a 1% cholesterol diet (Harlan Teklad; catalog # TD 89109). After 1 month on the 1% cholesterol diet, the rabbits were bled after an overnight fast. Plasma cholesterol levels were again determined, and groups of three rabbits were selected with similar plasma cholesterol levels. Each rabbit from the trio was then randomly assigned to one of three treatment groups. The 1% cholesterol diet was continued, and the rabbits received daily subcutaneous injections in the loose skin (the nape) of the neck of 2 ml of vehicle alone (50 mM ammonium bicarbonate, pH 7.0, containing 0.1 mg/ml Tween-20) or vehicle containing D-4F or L-4F at 20 mg/ml and administered at a dose of 10 mg/kg/day. After a month of daily subcutaneous injections, the rabbits weighed 3.75 ± 0.3 kg, and there was no significant difference in weight in any group, comparing the weight at the end of the study with the weight at the start of the study. There was no significant difference in water or food consumption among groups. The rabbits were bled after a 16 h fast and euthanized at the end of the study. The UCLA Animal Research Committee approved all aspects of the protocol.

Determination of aortic lesions

An en face method was used to determine rabbit aortic lesions as previously described (17). Briefly, after removing any adventitial fat, the heart was excised from each rabbit, with the attached and complete aortic tree, to just beyond the bifurcation to the common iliac arteries. The segment, containing short lengths of the innominate artery, the left common carotid, and the left subclavian artery, was bifurcated to expose the lumen for its entire length and through the aortic valve and left ventricle. The tissue was pinned flat on Styrofoam blocks and inverted into trays of 10% buffered formalin for overnight fixation and subsequent storage in sealed pouches for later analysis. The tissue was transferred to black wax trays and repinned and stained with Sudan IV. Photography was accomplished with a Canon 35 mm digital EOS SLR camera suspended beneath a tripod also mounting dual synchronized stroboscopic flash units. The area of the entire aorta and the area of Sudan IV staining were determined from the photographs in a blinded fashion and in triplicate using the Image Pro Plus software program (Media Cybernetics, Inc.; Silver Spring, MD). Triplicate measurements yielded a mean coefficient of variation of 17.34%. The average value for each specimen from the three determinations was used for group analysis.

Lipoprotein inflammatory index

Rabbit plasma was obtained from heparinized blood and was sucrose-cyropreserved by adding 20% by volume of a 5× stock of filter-sterilized 50% w/v sucrose, 150 mM NaCl, 0.24 mM EDTA, pH 7.4, in pyrogen-free water. The plasma was frozen and stored at −80°C until it was separated by fast protein liquid chromatography (FPLC) as previously described (11). Briefly, the column was eluted with an isocratic buffer containing 154 mmol/l NaCl and 0.02% sodium azide, pH 8.2, at a flow rate of 0.5 ml/min, pumped by a nonmetallic Beckman HPLC pump. Forty-eight 1 ml fractions were collected. Cholesterol concentrations were determined in fractions 13–36 to get a complete profile of the void volume through the post-HDL fractions. The cholesterol profile was used to identify the LDL and HDL regions. For LDL, fractions 18–23 or fractions 19–24 were pooled, depending on the FPLC cholesterol profile. For HDL, fractions 25–31, 25–32, or 26–32 were pooled, depending on the cholesterol profile. ApoB was not found in the HDL fractions but was found in the LDL fractions, as determined by Western blot, using a goat polyclonal antibody to rabbit apoB (Abcam, Inc.; Cambridge, MA), which was detected with an anti-goat biotinylated IgG secondary antibody (Vector Laboratories; Burlingame, CA), and ECL+ was used according to the manufacturer's instructions (Amersham Biosciences/GE Health Care; Piscataway, NJ) for visualization on a Typhoon 9410 Variable Mode Imager (Molecular Dynamics/GE Health Care). The LDL and HDL fractions were collected and tested in cultures of human aortic endothelial cells as described previously (11). Briefly, a standard control human LDL, prepared by ultracentrifugation of the plasma of a healthy volunteer, was added as an internal standard to all cultures at a concentration of 100 μg/ml cholesterol. After 8 h, the supernatants were collected and the monocyte chemotactic activity (which is largely due to the activity of MCP-1) in the supernatant was determined as previously described (8, 18). The values for the standard control LDL were normalized to 1.0. For determination of the HDL-inflammatory index (HII), a standard control human HDL, prepared by ultracentrifugation of the plasma of a healthy volunteer, or the test rabbit HDL, prepared by FPLC, was added at 50 μg/ml cholesterol, together with the standard control human LDL at 100 μg/ml cholesterol. Monocyte chemotactic activity was measured as migrated monocytes per high-powered field, in triplicates in nine separate fields, after incubation of the endothelial cells with the lipoproteins. The value obtained by addition of the standard control human LDL with the test HDL was divided by the monocyte chemotactic activity obtained after adding the standard control human LDL to the endothelial cells without HDL. In this assay, anti-inflammatory HDL results in HII values <1.0 and pro-inflammatory HDL results in HII values >1.0. For determination of the LDL-inflammatory index (LII), the test rabbit LDL was added to the cells at 100 μg/ml cholesterol without added HDL, and the resulting monocyte chemotactic activity was divided by the monocyte chemotactic activity obtained after addition of the standard control human LDL at 100 μg/ml cholesterol without added HDL. In this assay, if the test LDL induces more monocyte chemotactic activity than the standard control human LDL, the LII value will be >1.0. Conversely, if the test LDL produces less monocyte chemotactic activity than the standard control human LDL, the LII value will be <1.0.

SAA

SAA levels were determined using a non-species-specific SAA ELISA kit (Tridelta Development Limited, Bray, County Wicklow, Ireland; obtained through Tri-Delta Diagnostics, Inc., Morris Plains, NJ). Briefly, samples, including standards of known SAA content, were added to precoated microwells containing a captured monoclonal antibody specific for SAA, together with biotinylated anti-SAA monoclonal antibody. SAA present in the well

was both captured on the plate by the immobilized antibody and labeled with the conjugate antibody in a one-step procedure. After washing to remove all of the unbound material, streptavidin-horse radish peroxidase conjugate was added to the wells and incubated. Following the second incubation, tetramethyl benzidine substrate solution was added for 30 min at room temperature, at which time an acidic stop-solution provided by the manufacturer was added, and the intensity of the resulting color was determined in a spectrophotometer at 450 nm.

In vitro incubations with peptides

Peptide activity on apoB-containing lipoproteins in vitro was tested by incubating D-4F, L-4F, or scrambled L-4F at 10 μg/ml (the predicted C_{max} for the doses used in vivo) with pooled plasma samples from fasted rabbits that had been on the 1% cholesterol diet for 1 month but prior to the initiation of the treatment regimens. The samples were incubated for 1 h at 37°C on Nutator (providing gentle mixing). Four treatment conditions were compared: a no-treatment sham addition (vehicle alone), scrambled L-4F (10 μg/ml), D-4F (10 μg/ml), or L-4F (10 μg/ml). Triplicate incubations were performed. FPLC fractionation of 200 μl aliquots of the incubated plasma were performed on three sets of dual Superose 6 columns. Each treatment condition was fractionated on each set of columns and their respective pumps so as not to bias the study by system differences. Cholesterol determinations were performed on the resultant fractions. Internal controls were utilized to normalize the cholesterol data obtained from the twelve 96-well plates, each with its own standard curve. Normalized data were graphed as individual profiles. The standard deviations were calculated and plotted as best-fit smooth lines in Excel.

Cholesteryl ester transfer protein activity

Plasma cholesteryl ester transfer protein (CETP) activity was measured using a kit (KT-149) from Kamiya Biomedical Co. (Seattle, WA), following the manufacturer's instructions. Briefly, the assay uses a donor molecule containing fluorescent self-quenched neutral lipid that is transferred to an acceptor molecule in the presence of CETP. CETP-mediated transfer of the fluorescent neutral lipid to the acceptor molecule results in an increase in fluorescence (excitation, 465 nm; emission, 535 nm). The specific CETP activity of the sample is calculated as pmol/μl/h.

Other methods, including statistical analyses

Plasma lipid and lipoprotein concentrations were determined as previously described (12, 18), and statistical analyses were performed by ANOVA or unpaired two-tail *t*-test. Regression and correlation calculations were all made using GraphPad InStat, version 3.05, 32 bit for Windows 95/NT (GraphPad Software; San Diego, CA).

RESULTS

Plasma lipid, lipoprotein levels, and CETP activity

Table 1 contains the plasma cholesterol levels for the three groups prior to starting the experiment, after 1 month on the 1% cholesterol diet (i.e., just prior to treatment), and after 2 months on the cholesterol diet (i.e., at the conclusion of the treatment period). As shown in Table 1, the three groups were well matched for plasma cholesterol levels prior to the treatment period (i.e., there were no significant differences among groups prior to treatment). As expected, there was a significant increase in plasma cholesterol levels after 1 month on the 1% cholesterol diet. In the vehicle group, there was an approximately 19% significant decline in plasma cholesterol levels after 2 months on the diet, compared with after 1 month on the diet (Table 1). There was an even larger decrease in plasma cholesterol levels after treatment with D-4F (an approximately 36% decrease), which was highly significant (Table 1). Similarly, there was an approximately 33% decrease after treatment with L-4F, which was also highly significant (Table 1). Although the values after 1 month on the diet were not significantly different among groups, the difference between the D-4F and L-4F groups compared with the vehicle-treated group was significant after 2 months on the diet (i.e., after 1 month of treatment with vehicle or the peptides) (Table 1). As shown in **Fig. 1A**, B, peptide treatment shifted the FPLC cholesterol profile of the apoB-containing lipoproteins to smaller-sized particles. Incubation of the peptides in vitro with pooled plasma from fasted rabbits after 1 month on the 1% cholesterol diet but before treatment was started did not cause any significant changes in the FPLC cholesterol profile of the apoB-containing lipoproteins (data not shown), suggesting that the change in profile seen in vivo was not due to a direct action of the peptides on the apoB-containing lipoproteins. Although there was a significant decrease in plasma cholesterol in the apoA-I mimetic peptide-treated rabbits (Table 1), there was a significant increase in plasma triglycerides (**Table 2**). The triglyceride values presented in Table 2 were determined on whole plasma. Analysis of FPLC fractions 13–18 (VLDL fractions) revealed an increase in triglycerides in these fractions consistent with the increase in the whole plasma (data not shown).

TABLE 1. Plasma cholesterol levels

Treatment Group	Prior to 1% Cholesterol Diet	After 1 Month on 1% Cholesterol Diet but Before Treatment	After 2 Months on 1% Cholesterol Diet and After 1 Month of Treatment
		mg/dl	
Vehicle	60 ± 14	1,963 ± 515[d]	1,591 ± 382[a]
D-4F	65 ± 19	2,018 ± 790[d]	1,292 ± 317[b,e]
L-4F	53 ± 15	1,701 ± 626[d]	1,148 ± 247[c,f]

[a] $P < 0.05$, after 2 months on diet versus after 1 month on diet.
[b] $P < 0.001$, after 2 months on diet versus after 1 month on diet.
[c] $P < 0.01$, after 2 months on diet versus after 1 month on diet.
[d] $P < 0.001$, after 1 month on diet versus prior to diet.
[e] $P < 0.05$, after 2 months D-4F versus vehicle.
[f] $P < 0.01$, after 2 months L-4F versus vehicle.

There were no significant differences among groups in plasma HDL-cholesterol levels after 2 months on the 1% cholesterol diet and after 1 month of treatment. HDL-cholesterol levels at the conclusion of treatment were 34 ± 12, 33 ± 25, and 24 ± 10 mg/dl, respectively, for vehicle, D-4F, and L-4F. Similarly, there were no significant differences among groups in plasma CETP activity after 2 months on the 1% cholesterol diet and after 1 month of treatment. CETP activities at the conclusion of treatment were 17.8 ± 6.2, 18.7 ± 12.8, and 18.4 ± 5.12 pmol/μl/h, respectively, for vehicle, D-4F, and L-4F.

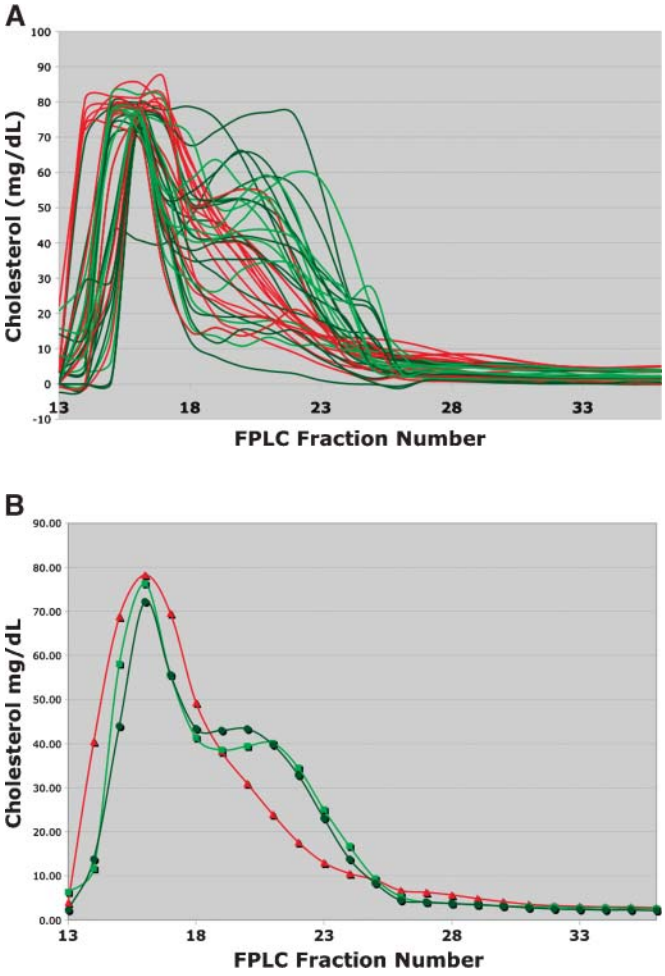

Fig. 1. Peptide treatment resulted in a shift of the cholesterol profile for the apolipoprotein B (apoB)-containing fast protein liquid chromatography (FPLC) fractions to smaller-sized particles. At the end of treatment, plasma from each rabbit was fractionated by FPLC, and the cholesterol concentration of each fraction was determined as described in Materials and Methods. Western blotting performed as described in Materials and Methods confirmed that the shifted FPLC fractions contained apoB (data not shown). A: The FPLC cholesterol profile from each rabbit is shown (red lines, vehicle-treated rabbits, n = 15; bright green lines, D-4F-treated rabbits, n = 15; dark green lines, L-4F-treated rabbits, n = 15). B: The average of each FPLC fraction from each treatment group is shown. Red, vehicle-treated; bright green, D-4F-treated; dark green, L-4F-treated.

Lipoprotein inflammatory indexes

Figure 2 demonstrates that after 2 months on the 1% cholesterol diet and 1 month of treatment with vehicle, the rabbit HDL was pro-inflammatory (i.e., it significantly increased the monocyte chemotactic activity resulting from the addition of a standard control human LDL to human aortic endothelial cells in culture, yielding an HII >1.0). In contrast, the rabbits treated with the apoA-I mimetic peptides had values for HII indicating anti-inflammatory HDL (i.e., the HDL significantly decreased the monocyte chemotactic activity resulting from the addition of a standard control human LDL to human aortic endothelial cells in culture, yielding an HII <1.0). There was no significant difference in HII between rabbits treated with D-4F or L-4F.

TABLE 2. Plasma triglyceride levels

Treatment Group	Prior to 1% Cholesterol Diet	After 1 Month on 1% Cholesterol Diet but Before Treatment	After 2 Months on 1% Cholesterol Diet and After 1 Month of Treatment
		mg/dl	
Vehicle	18 ± 7	19 ± 7	39 ± 15[a]
D-4F	20 ± 14	51 ± 115	324 ± 213[b,d]
L-4F	17 ± 9	21 ± 12	170 ± 162[c,e]

[a] $P < 0.001$, vehicle after 2 months compared with after 1 month and prior to diet.
[b] $P < 0.001$, D-4F after 2 months compared with after 1 month and prior to diet.
[c] $P < 0.001$, L-4F after 2 months compared with after 1 month and prior to diet.
[d] $P < 0.001$, D-4F vs vehicle after 2 months on diet.
[e] $P < 0.001$, L-4F vs vehicle after 2 months on diet.

Figure 3 demonstrates that after 2 months on the 1% cholesterol diet and 1 month of treatment with vehicle, the monocyte chemotactic activity following addition of the rabbit LDL was greater than the monocyte chemotactic activity induced after the addition of a standard control human LDL to human aortic endothelial cells in culture, yielding an LII >1.0. However, this increase in LII did not reach statistical significance. In contrast, addition of

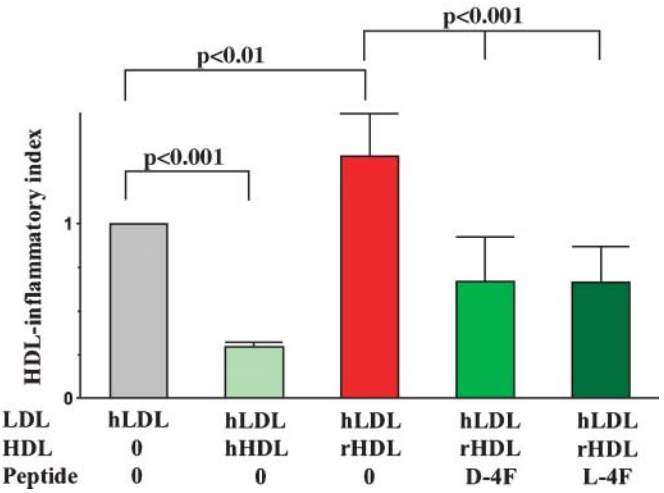

Fig. 2. HDL-inflammatory index (HII) was significantly improved after treatment with apoA-I mimetic peptides. A standard control human LDL (hLDL) was added to cultures of human aortic endothelial cells at a concentration of 100 μg/ml cholesterol. Eight h later, the culture supernatants were removed, and the monocyte chemotactic activity in the supernatants was determined as described in Materials and Methods. The values for the monocyte chemotactic activity following addition of the standard control human LDL without added HDL (gray bar) was normalized to 1.0. Other wells received the hLDL at 100 μg/ml cholesterol but also received HDL from a normal human (hHDL; pale green bar) or the test HDL from the rabbits (rHDL) at 50 μg/ml cholesterol from rabbits treated with vehicle (0 peptide; red bar) or D-4F (bright green bar) or L-4F (dark green bar). The resulting monocyte chemotactic activity was divided by that obtained from the addition of the hLDL alone to yield the HII, as described in Materials and Methods. The values shown are the mean ± SD, and n = 15 rabbits for each group.

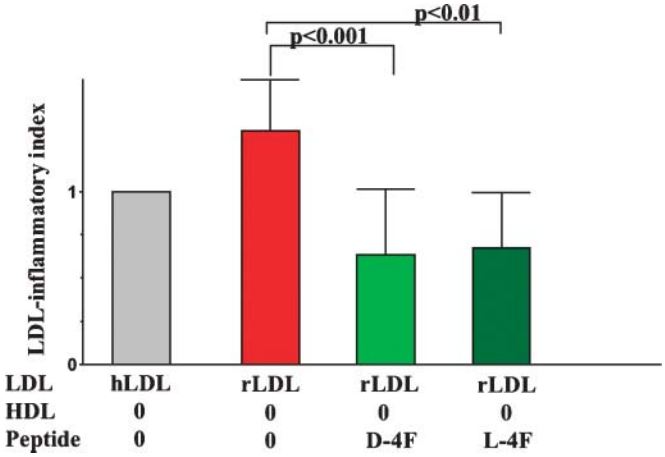

Fig. 3. LDL-inflammatory index (LII) was significantly improved after treatment with apoA-I mimetic peptides. A standard control human LDL (hLDL) was added to cultures of human aortic endothelial cells at a concentration of 100 μg/ml cholesterol. Eight h later, the culture supernatants were removed, and the monocyte chemotactic activity in the supernatants was determined as described in Materials and Methods. The values for the monocyte chemotactic activity following addition of the standard control human LDL without added HDL (0 HDL; gray bar) was normalized to 1.0. Other wells received rabbit LDL (rLDL) at 100 μg/ml cholesterol without added HDL (0 HDL) from rabbits treated with vehicle (0 peptide; red bar) or D-4F (bright green bar) or L-4F (dark green bar). The resulting monocyte chemotactic activity was divided by that obtained from the addition of the hLDL alone to yield the LII as described in Materials and Methods. The values shown are the mean ± SD, and n = 15 rabbits for each group.

LDL from rabbits treated with the apoA-I mimetic peptides induced significantly less monocyte chemotactic activity compared with that seen after the addition of a standard control human LDL to the cells, yielding an LII <1.0. There was no significant difference in LII between rabbits treated with D-4F or L-4F (Fig. 3).

Aortic lesions

As shown in **Fig. 4**, treatment with the apoA-I mimetic peptides significantly reduced aortic atherosclerotic lesions compared with the vehicle-treated group ($P < 0.01$ for D-4F compared with vehicle; $P < 0.05$ for L-4F compared with vehicle). None of the 15 rabbits treated with vehicle had less than 10% of aorta with lesions; whereas approximately half of the peptide-treated rabbits had less than 10% of aorta with lesions. There was no significant difference in the percent of aorta with lesions in rabbits treated with D-4F or L-4F (Fig. 4).

SAA

SAA is a well-described and well-accepted positive acute-phase protein that is a marker of systemic inflammation in rabbits (19). As shown in **Fig. 5**, there was a dramatic difference in SAA levels in the three treatment groups. The values (mean ± SD) for SAA were 95 ± 39, 8 ± 22, and 7 ± 19 μg/ml, respectively, for rabbits treated with vehicle, D-4F, or L-4F ($P < 0.001$ for peptide-treated rabbits compared with vehicle-treated rabbits). None of the

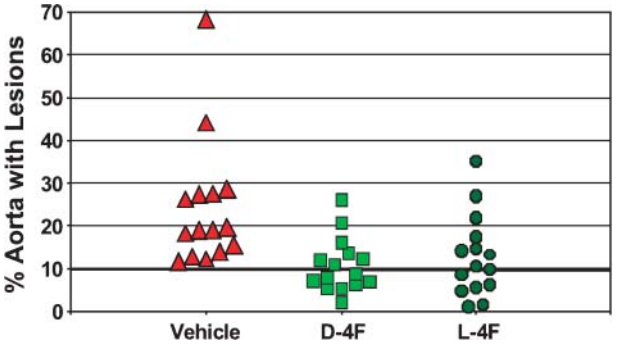

Fig. 4. Aortic atherosclerotic lesions were significantly decreased by treatment with apoA-I mimetic peptides. New Zealand White rabbits were fed a 1% cholesterol diet for 1 month. The diet was continued and the rabbits were given daily subcutaneous injections of vehicle (red triangles) or vehicle containing D-4F (bright green squares) or L-4F (dark green circles) at 10 mg/kg/day. After 1 month of treatment, the rabbits were euthanized, and the percent of aorta with atherosclerotic lesions was determined as described in Materials and Methods. The values shown are for 15 rabbits in each group. $P < 0.01$ for D-4F compared with vehicle; $P < 0.05$ for L-4F compared with vehicle.

15 vehicle-treated rabbits had SAA levels below 20 μg/ml, whereas only 1 out of 15 rabbits treated with D-4F and 1 out 15 rabbits treated with L-4F had values above 20 μg/ml (Fig. 5).

Correlation of percent of aorta with lesions, plasma lipids, lipoproteins, lipoprotein inflammatory indexes, and SAA levels

Despite the greater decrease in plasma cholesterol levels after treatment with the apoA-I mimetic peptides compared with treatment with vehicle (Table 1), there was no correlation between total plasma cholesterol levels and the percent of aorta with atherosclerotic lesions among the three treatment groups (**Fig. 6A**). There also was no

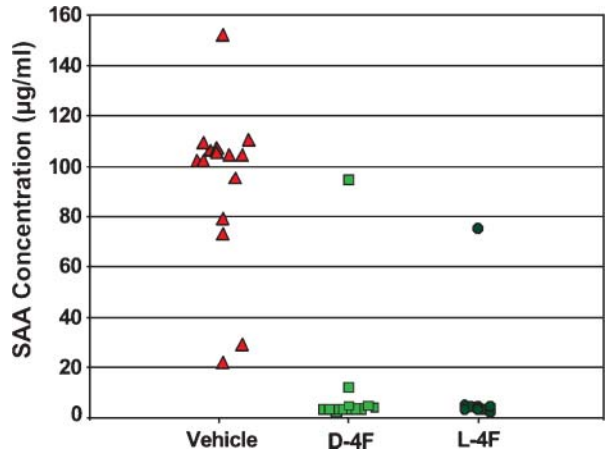

Fig. 5. Serum amyloid A (SAA) levels were dramatically reduced in peptide-treated rabbits. At the conclusion of treatment, SAA was measured as described in Materials and Methods. The values for the individual rabbits (n = 15 per group) are shown for the vehicle-treated (red triangles), D-4F-treated (bright green squares), and L-4F-treated (dark green circles) animals.

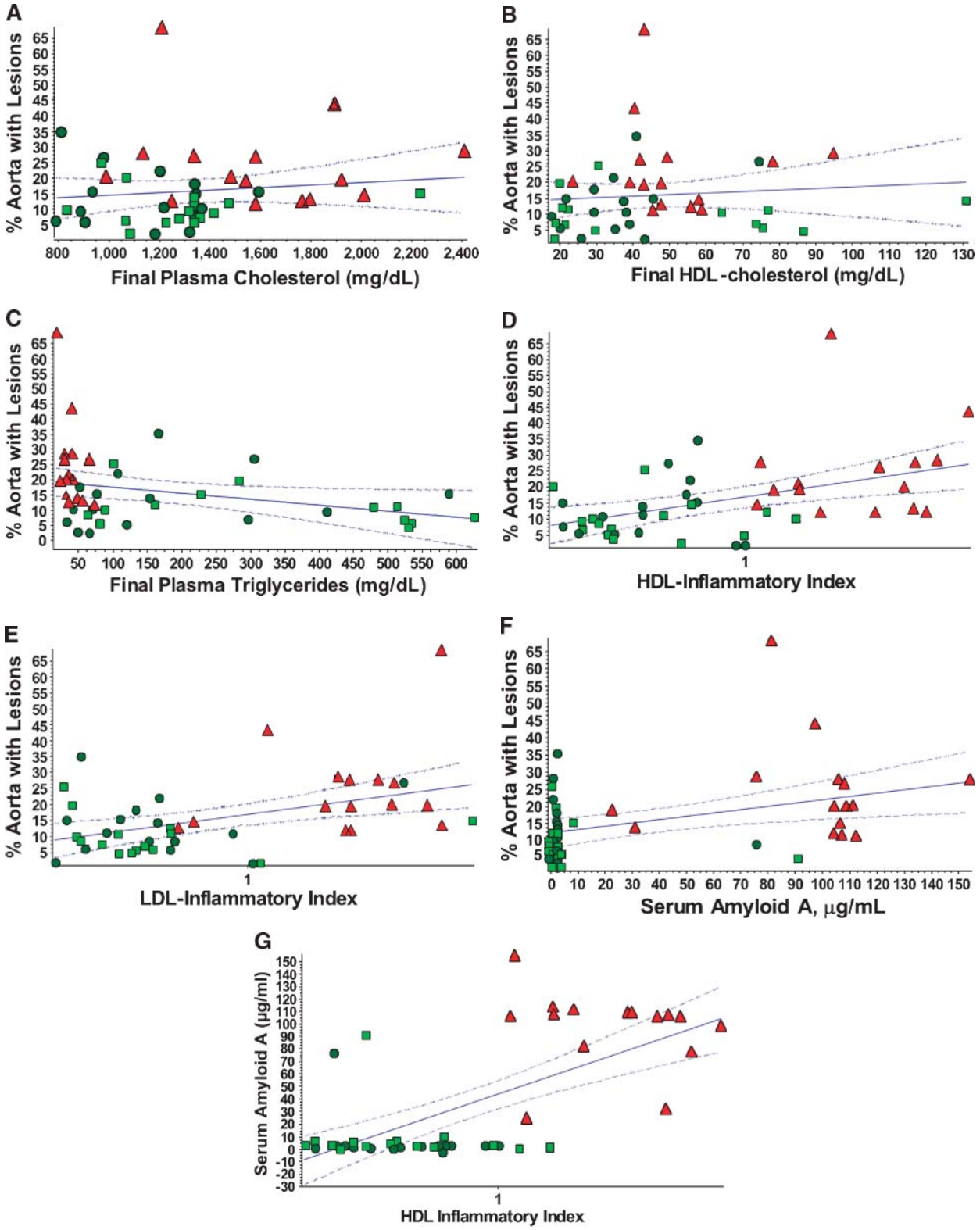

Fig. 6. Lipoprotein inflammatory properties and SAA levels were positively and significantly correlated with the percent of aorta with lesions and with each other; plasma triglyceride levels were inversely correlated with the percent of aorta with lesions; plasma cholesterol and HDL-cholesterol levels were not correlated with the percent of aorta with lesions. A–F: The correlation of the following parameters with the percent of aorta with lesions for the rabbits described in Fig. 4 was determined as described in Materials and Methods. The symbols are the same as in Figure 4 (i.e., vehicle, red triangles; D-4F, bright green squares; L-4F, dark green circles). A: Plasma cholesterol levels (r = 0.1245; r^2 = 0.01550; P = 0.4152). B: HDL-cholesterol levels (r = 0.09944; r^2 = 0.009888; P = 0.5207). C: Plasma triglyceride levels (r = −0.3096; r^2 = 0.09587; P = 0.0433). D: HII (r = 0.4494; r^2 = 0.2020; P = 0.0020). E: LII (r = 0.4390; r^2 = 0.1927; P = 0.0026). F: SAA levels (r = 0.3910; r^2 = 0.1529; P = 0.0079). G: The correlation between SAA levels and HII was determined as described in Materials and Methods. Symbols are the same as in A–F) (r = 0.6616; r^2 = 0.4377; $P < 0.0001$).

correlation between the percent of aorta with lesions and HDL-cholesterol levels (Fig. 6B). Interestingly, there was a significant inverse correlation between plasma triglyceride levels and the percent of aorta with atherosclerotic lesions (Fig. 6C). Although there was no relationship between HDL-cholesterol levels and the percent of aorta with lesions (Fig. 6B), there was a highly significant correlation between the HII and the percent of aorta with lesions (Fig. 6D). Figure 6D shows that there was clustering of the values for the vehicle-treated and peptide-treated groups, respectively, with no difference between the rabbits treated with D-4F or L-4F. Similarly, although there was no relationship between total plasma cholesterol levels and the percent of aorta with lesions (Fig. 6A), there was a highly significant correlation between the LII and the percent of aorta with lesions (Fig. 6E). There was also a highly significant correlation between SAA levels and the percent of aorta with lesions (Fig. 6F) and a highly significant correlation between SAA levels and HII (Fig. 6G). The data in Fig. 6G show strong clustering of the values for the vehicle-treated and peptide-treated groups, respectively, with no difference between the rabbits treated with D-4F and L-4F.

DISCUSSION

The data presented in Fig. 6 strongly suggest that the inflammatory properties of lipoproteins are a reflection of systemic inflammation and, together with SAA, are better correlated with the extent of atherosclerosis in the cholesterol-fed rabbit than are plasma cholesterol levels. Although the inflammatory indices of the lipoproteins and SAA levels correlated better with lesion area than the plasma lipoprotein levels did, we do not exclude a contribution to the decrease in lesion area with peptide treatment, inasmuch as there was a significant decrease in apoB-containing lipoproteins with peptide treatment compared with vehicle treatment.

These data constitute the first direct evidence that lipoprotein inflammatory properties are predictive of lesion area in an animal model. These data further establish the relationship between inflammation as measured by SAA levels and lesion area in cholesterol-fed rabbits and are consistent with the results of Lewis et al. (20), who found that SAA levels (but not plasma cholesterol levels) predicted lesion area in LDL receptor-null mice fed a high-cholesterol, high-fat diet. SAA has been implicated in atherosclerosis both as a marker of inflammation and perhaps as a direct contributor to lesion formation (20, 21). We previously reported that SAA increased in HDL in rabbits on a chow diet during an acute-phase reaction induced by croton oil or in humans undergoing a surgical procedure, and the increased content of SAA in HDL was associated with a conversion of HDL from anti-inflammatory to pro-inflammatory (19). In a subsequent study, we observed that HDL from patients with coronary heart disease failed to inhibit LDL-induced MCP-1 in cultures of human artery wall cells despite the fact that their SAA levels were not different from controls, strongly suggesting that the inflammatory properties of HDL cannot be solely determined by SAA (22). Thus, we think it likely that SAA is a marker of inflammation that is significantly correlated with the HII (Fig. 6G), but SAA is not the sole explanation for pro-inflammatory HDL. Similarly, we have previously demonstrated that high-sensitivity C-reactive protein cannot be the explanation for pro-inflammatory HDL (23). The molecular explanation for pro-inflammatory HDL remains under investigation, but in mice, the mechanism appears in part to be related to the association of hemoglobin with HDL under conditions of atherosclerosis/hyperlipidemia (24). A number of proteins with pro-inflammatory HDL have been identified using a proteomics approach (25, 26). The bioassay used to identify pro-inflammatory HDL in our current studies is based on the ability of the HDL to inhibit LDL-induced MCP-1 (27), as measured by a bioassay that measures monocyte chemotactic activity in cultures of human aortic endothelial cells. We also measured the ability of rabbit LDL to induce monocyte chemotactic activity compared with a standard control human LDL. These and similar assays were used in the initial selection of 4F as an apoA-I mimetic peptide for study in animal models (28).

The ability of class A amphipathic helical peptides to interact with nonoxidized lipids does not accurately predict their anti-atherogenic potential (29). The most important characteristics for predicting the ability of these peptides to inhibit atherosclerosis in animal models are the ability to *i*) interact with membranes (or the surfaces of lipoproteins) and remove oxidized lipids and *ii*) significantly decrease LDL-induced monoctye chemotactic activity in cultures of human aortic wall cells (29–34). The highly significant reduction in HII (Fig. 2) and LII (Fig. 3) after treatment with these apoA-I mimetic peptides is consistent with a mechanism involving the binding of oxidized lipids (11, 29–34) and with the previously reported presence of these oxidized lipids in lesions from cholesterol-fed rabbits (35) and the ability of oxidized lipids to accelerate atherosclerosis in this animal model (36).

We previously reported that oral administration of 4F synthesized from all L-amino acids (L-4F) resulted in degraded peptide in the plasma and no significant effect on HDL or LDL inflammatory properties (8). In contrast, administration of 4F synthesized from all D-amino acids resulted in significant improvement in the inflammatory properties of both HDL and LDL (8). The failure of L-4F was thought to be due to its degradation. Remaley et al. (37) reported that a peptide containing two class A amphipathic helixes (37pA) was not effective if synthesized from a mixture of D- and L-amino acids but was equally effective in interacting with ABCA1 if synthesized from all D- or L-amino acids. Tang et al. (38) reported that L-4F interacted with ABCA1 in vitro and confirmed that the interaction of class A amphipathic helical peptides in vitro with ABCA1 was independent of whether the peptides were synthesized from D- or L-amino acids. Ou et al. (10) demonstrated that L-4F given by intraperitoneal injection was effective in vivo in mouse models in improving

vasodilation. To our knowledge, this report is the first to directly compare the efficacy of a class A amphipathic helical peptide synthesized from all L-amino acids with the same peptide synthesized from all D-amino acids on lesion formation in an animal model.

We did not determine the pharmacokinetics of D-4F compared with L-4F in these studies. Garber et al. (39) reported on the tissue distribution and excretion of the peptide 18A. This peptide differs from 4F in that 18A does not have blocking groups as does 4F, and in 4F, two of the leucines on the hydrophobic face of the peptide have been changed to phenylalanines. The 18A peptide has many of the same binding characteristics for nonoxidized lipids as does 4F, but the 18A peptide is inactive in vivo in mouse models of atherosclerosis, in contrast to the dramatic efficacy of 4F.

The tissue distribution and excretion of ^{125}I-labeled 18A synthesized from L-amino acids (L-18A) was compared with the peptide synthesized from all D-amino acids (D-18A). The peptides were administered to rats by venous injection and the plasma concentrations and tissue distribution determined, the latter 3 days after injection. Most of the labeled peptide in plasma appeared in HDL after injection of either L-18A or D-18A.

The clearance from plasma was not significantly different for L-18A compared with D-18A. Three days after injecting L-18A, most of the radiolabel was lost from the rats, and what remained was recovered in the thyroid. Presumably, the peptide was degraded in the plasma and tissues, and the ^{125}I-labeled amino acids that were not excreted in the urine were concentrated in the thyroid ($94 \pm 0.4\%$). Only $5 \pm 0.56\%$ of the radioactivity that was injected was found in the rats 3 days after injection of L-18A. In contrast, $44.2 \pm 2.18\%$ of the radioactivity remained in the rats 3 days after injecting D-18A, and only $0.34 \pm 0.07\%$ of the radioactivity was found in the thyroid. To compare the distribution of the D and L peptides, the organ distribution of radioactivity excluding the thyroid was determined. Most of the radioactivity from both L-18A and D-18A was found in the liver and kidney. Because free ^{125}I did not appear in plasma or urine after injection of D-18A (in contrast to the case after injection of L-18A), it was concluded that D-18A remained in the tissues undegraded 3 days after injection (39). Thus, it is likely that in the case of peptides synthesized from all D-amino acids, some nondegraded peptide will remain in tissues for an undetermined prolonged period.

The fact that apoA-I mimetic peptides synthesized from either D- or L-amino acids were equally effective suggests that their mechanism of action is related to their ability to bind lipids and not to their ability to interact with proteins. However, this is not a certainty, inasmuch as there are now many examples of nonstereoselective protein-protein interactions, such as the pattern recognition receptors. Often these types of interactions are mediated by hydrophobic instead of ionic interactions.

As was the case in mouse models of atherosclerosis (8), treatment with these apoA-I mimetic peptides did not significantly alter HDL-cholesterol levels. However, contrary to the case for the oral administration of D-4F in mouse models of atherosclerosis (8), subcutaneous injection of these apoA-I mimetic peptides did significantly reduce plasma cholesterol levels and significantly increased plasma triglyceride levels in these cholesterol-fed rabbits (Tables 1 and 2). These differences could be due to the different routes of administration, with the subcutaneous route producing much higher plasma concentrations than would be expected following an oral dose (11).

It should be noted that there was a highly significant increase in plasma triglycerides in the vehicle-treated group, but the magnitude of the increase was significantly less than in the apoA-I mimetic peptide-treated groups. The reports by Miller and colleagues (40, 41) that infusion of lipid-free human apoA-I or infusion of human apoA-I with phosphatidylcholine discs into humans increased plasma triglycerides and the infusion of the latter reduced plasma apoB concentrations may be relevant, because the peptides studied here are apoA-I mimetics.

We hypothesize that the 4F peptide has both anti-inflammatory and antioxidant properties. In studying a rat model of diabetes, Abraham and colleagues (16, 42) discovered that 4F induces the antioxidant enzymes heme oxygenase-1 (HO-1) and extracellular superoxide dismutase and dramatically reduces superoxide anion levels in vascular tissue. Similarly, in a mouse heart transplant model, 4F was shown to induce HO-1 and inhibit chronic rejection (15). The work of Pritchard and colleagues (43) demonstrated similar results, in that they found that 4F treatment prevented the LDL-induced shift in the production of superoxide anion relative to nitric oxide production by endothelial nitric oxide synthase. More recently, Pritchard and colleagues (44) reported that 4F significantly reduced oxidative stress in Tight-skin mice. We previously published that 4F dramatically reduced lipoprotein lipid hydroperoxide content in apoE-null mice (11). Thus, there are considerable data to suggest that 4F is both anti-inflammatory and antioxidant. Administration of two quite different potent antioxidants in cholesterol-fed rabbits reduced aortic lesion area but paradoxically increased triglycerides (45, 46). We do not know the mechanism by which this occurs, but suspect that 4F acts by a similar mechanism. This effect appears to be restricted to cholesterol-fed rabbits, inasmuch as significant increases in triglycerides in mice treated with 4F in published studies have not been reported, and in unpublished studies in mice using even higher doses of peptide than used here, we have not seen a resulting increase in triglycerides (data not shown). Rabbits have a natural deficiency of hepatic lipase (47), and this may, in an as yet unidentified way, result in antioxidant-induced hypertriglyceridemia. Our in vitro studies suggest that there is no direct action of the peptides on apoB-containing lipoproteins. Also, peptide treatment in vivo did not alter CETP activity. Therefore, the mechanism by which peptide treatment in cholesterol-fed rabbits resulted in a change in the FPLC profile of the apoB-containing lipoproteins and resulted in increased plasma triglyceride levels remains to be determined. There is a growing body of evidence that dysfunctional or pro-

inflammatory HDL may be predictive of chronic inflammatory diseases in general (1–7, 18, 23, 44, 48–50).

The work presented here also adds to a growing body of evidence that plasma and HDL cholesterol levels are poorly predictive of lesion area in animal models of atherosclerosis compared with measurements of inflammatory markers such as SAA and determinations of lipoprotein inflammatory properties. This work suggests that following lipoprotein inflammatory properties and markers of chronic inflammation during therapy potentially may improve outcomes in a variety of chronic inflammatory conditions. Prospective studies in humans will be required to test this hypothesis. Much is already known about the mechanism of action of the peptides used in the studies reported here (34). However, much is still unknown and will require considerable further work before the mechanism of action of these peptides is truly understood.

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