High-Density Lipoproteins Retard the Progression of Atherosclerosis and Favorably Remodel Lesions Without Suppressing Indices of Inflammation or Oxidation


Objective—Protective properties of high-density lipoproteins (HDL) may include reverse cholesterol transport and suppression of oxidation and inflammation. These were investigated in vivo, as were the effects of HDL on the characteristics of atherosclerotic lesions.

Methods and Results—Male apolipoprotein E knockout (apoE−/−) and apoE−/− mice expressing human apolipoprotein AI (hAI/apoE−/−) were studied up to 20 weeks after commencing a high-fat diet. Plasma HDL cholesterol was twice as high in hAI/apoE−/− mice. Over time, aortic root lesion area remained less in hAI/apoE−/− mice, although plaques became complex. In advanced lesions, plaque lipid was higher in apoE−/− mice, whereas plaque collagen and alpha actin were increased in hAI/apoE−/− mice. In nonlesional aorta, mRNA abundance for pro-inflammatory proteins (vascular cell adhesion molecule [VCAM]-1, intercellular adhesion molecule-1 [ICAM-1], monocyte chemoattractant protein-1 [MCP-1]) increased between 4 and 16 weeks in apoE−/− (but not wild-type) mice, and were not reduced by elevated HDL. Autoantibodies to malondialdehyde low-density lipoprotein (LDL) increased progressively in apoE−/− mice, with similar results in hAI/apoE−/− mice.

Conclusions—HDL retarded plaque size progression despite greatly elevated plasma cholesterol. This effect was over a wide range of lesion severity. Expression of hAI reduced plaque lipid and increased the proportion of plaque occupied by collagen and smooth muscle cells, but did not affect indicators of inflammation or LDL oxidation. (Arterioscler Thromb Vasc Biol. 2004;24:1904-1909.)

Key Words: high-density lipoproteins ■ mice ■ atherosclerosis ■ inflammation

Plasma high-density lipoprotein (HDL) cholesterol concentration is inversely related to the development of ischemic heart disease and its complications. Comparatively little is known, however, of exactly how HDL exerts this protection. In particular, it is not known whether higher plasma HDL alters plaque composition, or whether HDL simply retards temporally lesion progression.

A limitation to examining directly the effects of HDL in animal models has been the difficulty in sustaining the elevation of HDL. One successful approach has been the transgenic expression of human apolipoprotein A-I (hAI), which elevates HDL and retards the development of atherosclerosis in C57Bl6 mice fed atherogenic diet and in apolipoprotein E-deficient (apoE−/−) mice fed normal chow. Under the conditions in those studies, however, mice did not develop lesions beyond the foam cell stage, leaving open the question of the effects of HDL on the development of advanced lesions, more relevant to human atherosclerosis.

Rong et al have reported that elevating HDL retards the progression and favorably alters the composition of pre-existing complex lesions, whereas Shah et al have shown that injection of a single high dose of apoA-I Milano regressed established lesions in mice with advanced aortic atherosclerosis. Recently, decreased plaque size has been demonstrated in low-density lipoprotein (LDL) receptor-deficient mice in which HDL elevation was attained through adenovirus-mediated hAI expression. The present study in apoE−/− mice fed high-fat, high-cholesterol Western diet (WD) provides the first systematic examination of the quantitative effects of lifelong sustained elevation of HDL on the composition of lesions across a wide range of severity, and tests the efficacy of HDL to retard lesion progression in the face of higher levels of non-HDL cholesterol than previously studied.

Atherosclerotic plaques contain oxidized LDL (OxLDL) as recognized by antibodies against oxidatively modified modified phospholipids such as phosphatidylcholine oxidized at the 1- and 2-positions (PC(1,2-Ox) and PC(2,3-Ox), which are considered markers for LDL oxidation. These antibodies are therefore valuable tools for assessing the extent of LDL oxidation in vivo. In apolipoprotein B-desoxinactin mice, serum HDL cholesterol concentrations were significantly higher in hAI mice than the controls. This study shows that HDL can retard lesion progression in vivo and may favorably alter the composition of advanced lesions, suggesting that HDL can act directly on atherosclerotic plaques.
as malondialdehyde-LDL (MDA-LDL) and 4-hydroxynonenal-LDL (4-HNE-LDL). Furthermore, autoantibodies to MDA and 4-HNE correlate with the degree of atherosclerosis in mice. Two enzymes thought to be protective against oxidant stress are carried by HDL: platelet activating factor acetyl hydrolase and paraoxonase. In mice, transgenic expression of hAI results in a 4-fold increase in platelet activating factor acetyl hydrolase activity. Mice lacking paraoxonase show increased susceptibility to atherosclerosis, whereas adenovirus-mediated gene transfer of human platelet activating factor acetyl hydrolase to apoE−/− mice reduced atherosclerosis. Thus, we also investigated whether, in vivo, elevating HDL may reduce atherosclerosis by antioxidant mechanisms as reflected in the titers of autoantibodies to epitopes on OxLDL.

In addition, in vitro studies suggest an anti-inflammatory role for HDL. For example, HDL suppressed the cytokine-stimulated expression of vascular cell adhesion molecule-1 (VCAM-1), intercellular adhesion molecule (ICAM-1) and E selectin on cultured human umbilical ven endothelial cells. However, this was not confirmed in a study using human coronary artery endothelial cells. In male apoE/−/− mice maintained on a WD for up to 20 weeks. Although lesions became complex in both groups, elevation of HDL retarded the size progression of atherosclerosis, despite greatly elevated non-HDL cholesterol. Notably, these beneficial changes occurred without evidence of reduced LDL oxidation or aortic inflammation.

**Methods**

**Studies were approved by The Mount Sinai School of Medicine Animal Care and Use Committee. The apoE−/− and hAI/apoE−/− C57BL/6 mice we used have been described previously. Male apoE−/− (n = 6 per time point) and hAI/apoE−/− mice (n = 5 per time point, except at 20 weeks n = 3) were weaned at age 4 weeks and fed WD for 4, 8, 12, 16, and 20 weeks (Dyets Inc, Bethlehem, Pa). At euthanization, plasma was obtained and tissue was prepared for histological studies. In addition, male apoE−/− and hAI/apoE−/− mice (n = 4 to 5 per group per time point) were fed WD for 8, 12, or 16 weeks before harvesting the thoracic aorta for mRNA measurements. Wild-type (WT) mice (n = 5) were fed WD as controls.**

For the following, please see http://atvb.ahajournals.org: measurement of plasma lipids and apoA-I; histology, immunohistochemistry, and morphometry; RNA extraction; quantification of mRNA using TaqMan real-time polymerase chain reaction; and assay for autoantibodies to MDA-LDL.

**Analysis of Plaque Composition**

Stained or immunostained areas on serial sections were quantified, as previously, on digitized images using the semi-automated hue analysis features in Image Pro Plus by an operator blinded to the origin of the slide. Because considerations of plaque stability are most relevant in advanced plaque, measurements were made on aortic sections from the 16-week and 20-week time points, which contained American Heart Association (AHA) type IV and V lesions. Areas of plaque that appeared green with combined Masson elastin (CME) stain (indicating the presence of collagen stained by the light green component15), and areas immunostained for α-actin were quantified.

During tissue processing in ethanol and xylene, 95% to 99% of plaque lipid is dissolved, leaving clear areas visible by light microscopy. As described previously, such transparent areas within plaque can be quantified to represent areas in which lipid was present before processing. These areas typically have needle-like forms, characteristic of crystals of cholesterol monohydrate. To validate this technique, we compared morphometric measurement of "lipid-free areas" at the aortic root with the biochemical measurement of aortic arch cholesterol isolated from the same mouse. For assay details, please see http://atvb.ahajournals.org.

**Statistical Methods**

Data are presented as mean±SD unless otherwise stated. Normally distributed parameters were compared by f tests and non-normally distributed data were analyzed by the Mann-Whitney test. Correlations were tested by Pearson method (GraphPad Prism, GraphPad Software Inc, San Diego, Calif). Statistical significance was P<0.05.

**Results**

**Plasma Cholesterol and ApoA-I Levels**

At the time of euthanization, non-HDL cholesterol (mg/dL) was similar in apoE−/− and hAI/apoE−/− male mice (973±353 versus 1196±365, respectively, P = NS). In contrast, HDL cholesterol was >2-fold higher in hAI/apoE−/− transgenic than apoE−/− mice (59±19 versus 27±8; P < 0.0001).

Human apoA-I was present in all hAI/apoE−/− transgenic mice (and none of three samples from apoE−/− mice). Plasma hAI (μg/mL) was 2300±670. Expression was sustained without diminution from weaning to 20 weeks afterward (data not shown). Mouse apoA-I was lower in hAI/apoE−/− (50±20) compared with apoE−/− (390±120) mice (P < 0.0001), but total apoA-I (the sum of mouse and human apoA-I) was higher in hAI/apoE−/− than apoE−/− mice (2350±670 versus 390±120, P = 0.0001). Thus, in hAI/apoE−/− mice, total apoA-I was elevated proportionally more than HDL cholesterol. Analysis of plasma fractionated by fast protein liquid chromatography (FPLC) showed hAI to be predominantly present in fractions corresponding to HDL (86%), with some (14%) in very-low-density lipoprotein (VLDL) fractions, implying increased apoAI per HDL particle in these mice.

**hAI Expression Retarded the Size Progression of Atherosclerosis and Altered Lesion Characteristics**

In the male animals studied here, at all time points, lesion area in the apoE−/− mice exceeded that in hAI/apoE−/− mice. At 8, 16, and 20 weeks, this was statistically significant, with a trend toward significance (P = 0.076) at the 12-week time point (Figure 1A). The difference increased from 4 to 20 weeks (R = 0.95, P < 0.01 for trend), suggesting a divergence in the rates of increase of lesion size between the groups. Despite the size retardation in the hAI mouse lesions, the complexity of the lesions became advanced in the 2 groups of mice (Figure 1B).

In validation experiments, cholesterol content of aortic arch (2.8 to 14.6 mg/mg tissue) correlated strongly (R = 0.99, P < 0.01) with the transparent areas in the CME-stained aortic root lesions (0.016 to 0.090 mm²), confirming that the morphometric technique reported faithfully reflects the cholesterol content of the aorta. Accordingly, transparent ("lipid-depleted") areas (mm²) within plaques that had been occupied by lipid before removal during tissue processing were >2-
fold higher in apoE−/− mice than hAI/apoE−/− mice (0.076±0.042 versus 0.037±0.027, P=0.015; Figure 2).

The proportion (%±SD) of plaque occupied by collagen was 2-fold higher in hAI/apoE−/− mice (31.46±8.9 versus 15.59±6.7, P=0.001; Figure 2). In addition, α-actin staining tended to be of greater relative abundance in the plaques of hAI/apoE−/− mice relative to apoE−/− controls, but over time lesions in both groups advanced in complexity.

Also at the 16- and 20-week time points, there was no difference in the areas of plaque staining positively with the monocyte/macrophage-specific antibody, MOMA-2 (0.047±0.05 versus 0.041±0.03 for apoE−/− and hAI/apoE−/−, respectively; P=NS; Figure 1A, available online at http://atvb.ahajournals.org). Significantly, in these relatively advanced plaques, macrophages were predominantly layered on the luminal aspect of the lesion and largely absent from necrotic deeper areas (Figure 1A). Shown in Figure 1B is staining for tissue factor, which colocalized to areas staining positively for macrophages, and which demonstrated similar staining characteristics to MOMA-2 (0.07±0.009 versus 0.05±0.01, P=NS).

Autoantibody Titers to MDA-LDL Increased in ApoE−/− and hAI/apoE−/− Mice
Autoantibody titers (IgM and IgG) to MDA-LDL were measured at time points shown in Figure 3. At weaning, levels (mean±SEM relative light units [RLU]) were comparable between apoE−/− (IgM, 51480±12080; IgG, 4470±1090) and hAI/apoE−/− mice (IgM, 64790±26110; IgG, 7690±2350). As shown, over time, IgM increased progressively in both apoE−/− (R=0.80, P<0.0001) and hAI/apoE−/− mice (R=0.87, P<0.0001). Notably, the rate

![Figure 1. hAI retards lesion growth. A, Atherosclerotic lesion size was greater in apoE−/− mice (black symbols) at all time points (tabulated data). The atheroprotective effects of transgene expression were sustained for 20 weeks with attenuation. The difference in mean lesion area increased with time (P<0.01), indicating divergence in the rates of lesion progression between the groups. B, Representative transverse sections of aortic root. At 8-week and 20-week time points, lesion size was significantly reduced in the hAI/apoE−/− transgenic mice relative to apoE−/− controls, but over time lesions in both groups advanced in complexity.](http://atvb.ahajournals.org/)

![Figure 2. hAI promotes changes associated with plaque stabilization. A, Mean lipid-depleted area assessed in 16-week and 20-week groups was 2-fold higher in apoE−/− mice (top) (n=12 for apoE−/−; black bar, and n=8 for hAI/apoE−/−; gray bar). The proportion of plaque occupied by collagen was 2-fold higher in hAI/apoE−/− mice (bottom) (gray bar). B, CME stains collagen green in lesions from apoE−/− (left) and hAI/apoE−/− mice (right). The computer-generated (ImagePro Plus) “mask” of transparent areas because of solvent extraction of lipid (bottom). The mask is generated from the CME-stained section of the upper panel. Attention is drawn to the high degree of correspondence between the white areas on the 2 panels. In both upper and lower panels, arrows indicate lipid-depleted areas, which possess the characteristic shape and distribution of cholesterol clefts.](http://atvb.ahajournals.org/)
and magnitude of increase of the IgM titers were similar between the 2 groups. IgG increased initially and then appeared to plateau, so that the positive correlation with duration of WD feeding, although still statistically significant, was less marked than that for the IgM (R = 0.61, P < 0.01 for apoE−/− and R = 0.38, P < 0.05 for hAI/apoE−/− mice).

Lesion area correlated with autoantibodies to MDA-LDL of the IgM class in both apoE−/− (R = 0.63, P < 0.001) and hAI/apoE−/− mice (R = 0.62, P = 0.01). There was no correlation between lesion area and levels of autoantibodies to MDA-LDL of the IgG class in either group.

**hAI Expression Did not Affect the Malondialdehyde and 4-Hydroxynonenal Contents of Atherosclerotic Lesions**

Immunostaining for MDA and 4-HNE was prominent in the lesions of both apoE−/− and hAI/apoE−/− mice and was predominantly associated with foam cells (Figure II, available online at http://atvb.ahajournals.org). There were no differences in the staining patterns in plaques from apoE−/− or hAI/apoE−/− mice. In the later time points (eg, 16 to 20 weeks), in addition to foam cell staining, there was weak staining in areas where the “lipid core” had been removed in tissue processing.

**hAI Does not Reduce VCAM-1, ICAM-1, or MCP-1 mRNA In Vivo**

After 4 weeks on WD, levels of mRNA for VCAM-1, ICAM-1, and MCP-1 were comparable in arterial samples taken from WT, apoE−/−, and hAI/apoE−/− mice. Thereafter, levels of each of these mRNAs increased in apoE−/− and hAI/apoE−/−, but not WT, mice. At all time points, levels of mRNA for VCAM-1, ICAM-1 and MCP-1 were similar between apoE−/− and hAI/apoE−/− mice (Figure 4). Compared with the mRNA levels measured at 4 weeks, at 16 weeks VCAM-1 (0.24 ± 0.075 versus 0.088 ± 0.014; P = 0.06), ICAM-1 (0.046 ± 0.01 versus 0.022 ± 0.004; P < 0.05), and MCP-1 (0.048 ± 0.007 versus 0.012 ± 0.004; P < 0.001) were all increased (n = 8 per group for all). Within individual mice, similar temporal patterns of increase of mRNA levels for VCAM-1, ICAM-1, and MCP-1 were noted, suggesting that these factors may be regulated in concert. Significant correlations were found between levels of ICAM-1 versus MCP-1 (R = 0.74, P < 0.0001), VCAM-1 versus MCP-1 (R = 0.64, P < 0.0003), and ICAM-1 versus VCAM-1 (R = 0.77, P < 0.0001).

**Discussion**

There are 2 major findings of these studies. First, transgenic expression of human apoA-I retarded the size progression of atherosclerosis in male apoE−/− mice consuming a WD, which caused marked elevations of non-HDL cholesterol. Notably, the protective effects on lesion size were sustained for the duration of the study. Significantly, the apparent
content of extracellular lipid in the lesions of apoE−/− mice was twice that in lesions of hAI/apoE−/− mice (reinforcing the importance of HDL in reverse cholesterol transport), whereas the proportion of plaque occupied by collagen was 2-fold higher in the hAI/apoE−/− mice. Thus, the lesions had features that in humans are associated with plaque stability. Although the size progression was retarded, it should be noted that the lesions in the hAI/apoE−/− animals still advanced past the foam cell stage, indicating separable quantitative and qualitative effects of HDL on plaques.

The other major finding of these studies is that the elevation of HDL to WT levels, despite the beneficial changes, did not suppress indices of oxidation or inflammation. Specifically, plasma titers of MDA-LDL autoantibodies and lesion immunostaining of MDA or 4-HNE were not reduced, nor were increases in the level of aortic mRNA of VCAM-1, ICAM-1, or MCP-1 suppressed.

**Effects on Lesion Characteristics**

Expression of hAI transgene has been shown to reduce lesion size in WT mice fed an atherogenic diet and in apoE−/− mice fed chow. In these earlier studies, hAI mice developed only foam cell lesions (AHA types I and II), which did not allow detailed analysis of the effects of HDL on plaque character. To our knowledge, no study has systematically examined the quantitative effects of HDL on plaque composition over a wide range of lesion classes, although there are recent data, consistent with our results, on plaque size changes in LDL receptor-deficient mice with adenovirus-mediated hAI expression.7

In the present study, by using human apoA-I transgenic mice, constant sustained elevation of HDL was achieved. Furthermore, the use of the WD in apoE−/− mice accelerated lesion formation such that effects of HDL on lesions of AHA classes I to V could be studied. The present work demonstrates the efficacy of HDL to reduce lesion size, despite greatly elevated non-HDL cholesterol. Note, however, that compared with previous studies, the magnitude of atheroprotection afforded by HDL was reduced, attributable to increased non-HDL cholesterol in the present study, consistent with observations in humans.20

In apoE−/− and hAI/apoE−/− mice fed chow diet for 8 weeks, HDL had no effect on the rate of subendothelial deposition of lipid.21 The current study found that HDL substantially reduced the areas of plaque that had been occupied by lipid before tissue processing. In combination, these findings suggest that evacuation of lipid from plaque is an important function of HDL.

Collagen, predominantly type I, is found in atherosclerotic plaque; its presence is associated with plaque stability.22 The present study has shown a substantial increase in hAI/apoE−/− mice in the proportion of plaque in advanced lesions (AHA types IV and V) that was occupied by collagen. Lesion staining for α-actin (a marker for vascular smooth muscle cells) also tended to increase in hAI/apoE−/− mice. In conjunction, the apparent reduction in extracellular lipid and the increases in collagen and smooth muscle cells suggest that HDL has contributed to the formation of a more stable type of plaque.

At early time points, lesions were largely or exclusively composed of foam cells (AHA lesion types I and II). Accordingly, because lesions were smaller in the hAI/apoE−/− mice, the areas of MOMA-2 staining were also smaller (data not shown). Progression of atherosclerosis in apoE−/− mice is accompanied by an increase in plaque complexity in which macrophage foam cells occupy proportionately less of the lesion.23 This may be a general phenomenon in mice because Steinberg et al have shown that, over time, both macrophage influx into lesions and cytokine responsiveness decrease.24 As shown in Figure I, in both apoE−/− and hAI/apoE−/− mice at the later time points macrophages were predominantly layered on the luminal aspect of the lesion and largely absent from necrotic deeper areas. This suggests ongoing progression of the atherosclerosis in sequential layers, as described previously in apoE−/− mice.23 This is also consistent with our current and previous conclusions that the protective effects of HDL are not mediated primarily by differences in monocyte recruitment, but by cholesterol removal and plaque remodeling. An alternative explanation for the reduced extracellular lipid accumulation in the hAI transgenic mice (without an alteration in macrophage content) is that HDL elevation may have reduced the rates of both macrophage accumulation and the necrosis of lipid-laden macrophages.

**Effects on Arterial Inflammation**

Elevating HDL in apoE−/− mice had no effect on the mRNA levels of VCAM-1, ICAM-1, or MCP-1 present in mouse thoracic aorta (in agreement with the VCAM immunohistochemistry on early aortic root lesions noted by Dansky et al21). Significantly, levels of each mRNA increased over time, but the magnitude and time course of these changes were similar in apoE−/− and hAI/apoE−/− mice. Within individual mice, levels of mRNA for VCAM-1, ICAM-1, and MCP-1 were correlated, suggesting that in the context of hyperlipidemia, these genes are regulated by common mechanisms. The 5′ flanking regions of VCAM-1, ICAM-1, and MCP-1 contain NF-κB consensus DNA-binding sites. This transcription factor activates a number of genes relevant to arterial wall pathology. NF-κB and its target genes are upregulated in animals at sites prone to atherosclerosis.28,29

**Effects on Autoantibodies to OxLDL Epitopes**

In apoE−/− mice fed WD, there was a time-related increase in autoantibodies to MDA-LDL. Increases were present for both IgM and IgG subtypes, but for IgM were more marked, and strikingly related to duration of feeding with WD. As previously described, the level of autoantibody correlated with the amount of atherosclerosis present in the aortic root. The expression of the hAI transgene, which increases the plasma concentration of HDL cholesterol and associated antioxidant defense systems, did not reduce the rate of increase or the levels of autoantibodies to MDA-LDL. In addition, MDA-LDL and 4-HNE were identified by immunohistochemistry in atherosclerotic plaque, but were unaffected by the presence of increased hAI and HDL. Therefore, by 2 separate measures (plasma autoantibodies to MDA-LDL and lesion MDA-LDL and 4-HNE), hAI expression did not appear to decrease significantly the formation of OxLDL in the lesions or the immune response to the neo-epitopes.
formed. It should be noted, however, that in this and other studies,\textsuperscript{5,14} there are possible limitations caused by a maximal immune response above a threshold of antigen burden or limits in the sensitivity of the standard measures used to detect changes in LDL oxidation and its sequelae. It should also be recognized that the substantial elevations of non-HDL cholesterol may have overwhelmed potentially subtle anti-inflammatory and antioxidant mechanisms, so that despite some beneficial effects of increased plasma HDL, lesions still advanced in complexity.

This study has shown the efficacy of HDL in retarding plaque size progression despite the presence of greatly elevated plasma cholesterol. The protective effects of HDL were observed across a wide range of lesion severity. Despite elevated HDL, and plaque retardation, lesions became complex and indices of inflammation and LDL oxidation were not suppressed. HDL reduced plaque lipid content and increased the proportion occupied by collagen and smooth muscle cells. If translated into humans, these effects may help to explain the benefits of HDL demonstrated in epidemiological studies.

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Figure IA

Bar graph showing MOMA-2 expression in ApoE-/- and hAI conditions. The graph indicates a significant difference between the two conditions, with ApoE-/- showing higher MOMA-2 expression compared to hAI.

Images below the graph illustrate the histological findings corresponding to the bar graph.
Figure IB
Figure II

MDA

4-HNE

ApoE<sup>−/−</sup>

hAl
**Online Methods- Choudhury et al.**

**Measurement of plasma lipids and apoA-I**

HDL was isolated from plasma by density gradient ultracentrifugation. Total cholesterol and HDL-C were analyzed with kits (Sigma Chemical Co). Human\(^1\) and mouse\(^2\) apoA-I were measured by ELISA, as described previously.

**Fast phase liquid chromatography (FPLC)**

For fractionation of plasma by FPLC, 400uL of fresh plasma, pooled from 3-4 mice (obtained after 12 weeks on diet) was filtered, injected onto two Superose 6 columns connected in series (FPLC system, Pharmacia Biotech, Piscataway, NJ.) and eluted at a constant flow rate of 0.3mL / min with 1mM EDTA and 0.15M NaCl. Ninety fractions of 0.3 mL were collected and cholesterol assayed, as described above.

**Histology, immunohistochemistry and morphometry**

Under anesthesia, mice were killed by exsanguinations / perfusion fixation with 4% paraformaldehyde in phosphate buffered saline, (pH 7.4). Serial 5µm sections were taken from paraffin-embedded aortic roots and stained with Combined Masson Elastin (CME) stain. Photomicrographs were analyzed using Image Pro\textsuperscript{®} Plus software (Media Cybernetics, Silver Spring, Maryland). The immunostaining protocol, use of negative controls and full antibody particulars are described elsewhere\(^3,4\). In addition, anti-MDA-LDL antibodies (Rabbit, 200ng / mL Alpha Diagnostics, San Antonio, TX) were used.
Analysis of plaque composition. Comparison of biochemical measurement and morphometric estimations of plaque cholesterol content.

Human apoAI/apoE deficient mice (n = 4) were fed a Western-type diet for 14 or 28 weeks to develop different extent of atherosclerotic lesions. At sacrifice, the mice were exsanguinated and perfused with PBS. The heart was removed, fixed in 4% paraformaldehyde, and embedded in paraffin. The aortic arch was removed and kept at minus 80°C. Adventitial fat was carefully removed from the aortic arch tissue under a dissecting microscope. During the process, deionized, distilled water was sprayed onto the tissue frequently to prevent dehydration. The arch tissue was then blotted dry, weighed (range 1.3 ~ 4.1 mg, depending on the extent of atherosclerosis), and homogenized in a Wheaton tissue grinder on dry ice. Lipid extraction was conducted using a modified Bligh-Dyer procedure. Briefly, PBS (0.2 ml) and chloroform:methanol (2:1, v/v; 1.2 ml) containing 0.1% butylated hydroxytoluene were added to the homogenized tissue, mixed vigorously, and centrifuged to separate the organic phase, which was collected and saved. The aqueous phase was re-extracted with 0.6 ml of chloroform:methanol solution, and the organic phases were pooled. The lipids in the organic phase were evaporated to dryness under nitrogen and redissolved in isopropanol. Free cholesterol content was determined using an R-Biopharm (49068 Marshal, MI) cholesterol kit following the manufacturer's instruction.

RNA extraction

Tissue for RNA quantification was not fixed; instead blood was removed by perfusion with PBS. The descending thoracic aorta was dissected, frozen in liquid nitrogen, and stored at -80°C pending RNA extraction. RNA was isolated with the
RNeasy Mini kit (Qiagen, Valencia, California). RNA samples were then treated with RNase-free DNase (Ambion, Austin, Texas) and quantified by a fluorescent assay (Ribogreen, Molecular Probes Inc, Eugene, Oregon). The choice of both (1) time points and (2) descending aorta were purposely designed to precede lesion development (since plaques express the mRNAs of interest in high quantities and differential lesion size would confound comparison between mouse groups), but to incorporate a period during which susceptible areas would be expected to up-regulate pro-inflammatory mediators.6

**Statistical Methods**

Data are presented as mean ± standard deviation unless otherwise stated. Normally distributed parameters were compared by t-tests and non-normally distributed data were analyzed by the Mann-Whitney test. Correlations were tested by Pearson’s method (GraphPad Prism, GraphPad Software Inc, San Diego, Ca.). Statistical significance was a P value <0.05.
References for on line Methods


