Elevating High-Density Lipoprotein Cholesterol in Apolipoprotein E–Deficient Mice Remodels Advanced Atherosclerotic Lesions by Decreasing Macrophage and Increasing Smooth Muscle Cell Content

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Background—HDL cholesterol levels are inversely correlated with coronary heart disease risk in humans, and in animal studies, HDL elevation decreases formation and progression of foam-cell lesions. The potential for HDL to affect preexisting advanced atherosclerotic lesions is not known. To approach this issue, we used a novel mouse aortic transplantation model.

Methods and Results—ApoE-deficient (EKO) mice were fed a Western-type diet for 6 months, and thoracic aortic segments containing advanced lesions replaced segments of the abdominal aorta of 4-month-old EKO syngeneic mice not expressing (plasma HDL cholesterol ≈26 mg/dL) or expressing (HDL ≈64 mg/dL) a human apoAI (hAI) transgene. Both types of recipients had comparable non-HDL cholesterol levels. Five months after transplantation, mice were killed and grafts analyzed. Compared with lesion area in pretransplant mice (0.14±0.04 mm², mean±SEM), there was progression in the EKO recipients (0.39±0.06 mm², P<0.01). Compared with EKO recipients, hAI/EKO recipients had retarded progression (0.24±0.04 mm², P<0.05). Immunostaining for CD68 and other macrophage-associated proteins, monocyte chemoattractant protein-1, acyl coenzyme A:cholesterol acyltransferase, and tissue factor, in lesions of pretransplant and EKO recipient mice showed abundant macrophages. In contrast, compared with any other group, lesional macrophage area in hAI/EKO mice decreased >80% (P<0.003), and smooth muscle cell content (α-actin staining) increased >300% (P<0.006). The decrease in macrophages and increase in smooth muscle cells was primarily in the superficial subendothelial layer.

Conclusions—Increasing HDL cholesterol levels in EKO mice retards progression of advanced atherosclerotic lesions and remodels them to a more stable-appearing phenotype. (Circulation. 2001;104:2447-2452.)

Key Words: muscle, smooth remodeling plaque lipoproteins

A large number of epidemiological studies have shown that the plasma level of either HDL cholesterol (HDL-C) or the major structural protein of HDL, apolipoprotein AI (apoAI), is inversely correlated with the risk of coronary heart disease.1 These epidemiological findings are supported by animal studies of the formation, progression, and regression of foam-cell lesions, which represent the earliest stage of atherosclerosis. For example, expression of a human apoAI (hAI) transgene in apoE-deficient (EKO) mice increased HDL-C to wild-type levels and significantly retarded lesion progression.2,3 In cholesterol-fed New Zealand White rabbits, intravenous injection of HDL particles4 led to regression of fatty streak lesions, and injection of apoAI2 foam-cell lesion progression. In LDL receptor–knockout mice fed a Western-type diet (WD), administration of a recombinant adenovirus containing hAI cDNA led to regression of foam-cell lesions.6 There are no data, however, from human or animal studies showing that a sustained and significant elevation of HDL would have beneficial effects on advanced, preexisting atherosclerotic lesions, an important issue given that by age 35 years, 79% of people have a significant lesion burden (with >40% having advanced lesions in their coronary arteries),7 and the recent speculation that such lesions have a limited capacity for improvement.8

Received June 22, 2001; revision received August 23, 2001; accepted August 27, 2001.

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Circulation is available at http://www.circulationaha.org

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Because of the proven efficacy of HDL to retard lesion formation in EKO mice transgenic for hAI (hAI/EKO), we asked whether the plasma lipoprotein environment in hAI/EKO mice would also promote beneficial changes in preexisting lesions that had advanced beyond the early foam-cell stage. The experimental approach is based on a novel mouse aortic transplantation model that we recently reported. In the present study, atherosclerosis was allowed to progress in EKO mice for 6 months, at which time aortic grafts containing advanced lesions were transplanted interpositionally into recipient mice with either the EKO or hAI/EKO genotype. In this way, the HDL level to which a lesion is exposed is rapidly and stably changed.

After 5 months in the 2 different lipoprotein environments, lesion characteristics were analyzed, revealing that the elevation of HDL levels resulted in quantitative and qualitative changes, including retarded progression of size, depletion of macrophage-derived foam cells and their proatherothrombotic products, and enrichment of intimal smooth muscle cells (SMCs). Thus, in addition to its ability to prevent macrophage-derived foam-cell formation, HDL may also have the potential to favorably remodel more advanced lesions to what would be considered in human plaques to be a more stable phenotype.

Methods

Experimental Design and Animals

The experimental design is summarized in Figure 1. Animal procedures were approved by the Institutional Animal Care and Research Advisory Committee. The EKO and hAI/EKO mice (C57BL/6 background) used in this study have been described before. EKO mice were weaned at 1 month of age onto a 21% (wt/wt) fat, 0.15% cholesterol “Western-type diet” (WD; catalogue No. 100244, Dyets Inc) and were fed this diet for 6 months to develop advanced thoracic aortic lesions containing necrotic lipid cores (Figure 2A) and SMCs in the cap (Figure 5A; AHA class IV). Mice were then divided into 3 groups. One group (pretransplant, n=5) was killed to obtain thoracic aortas for baseline analysis. A second group (mock donors, n=6) was switched to and maintained on a standard chow diet for 5 months and then killed to obtain data to control for nonspecific effects of the transplantation procedure. A third group (n=16) served as donors of thoracic aortic segments containing advanced lesions. The recipients of the segments were either EKO (n=7) or hAI/EKO (n=9) mice (4 months old) that were maintained on the standard chow diet before and after transplantation and were killed 5 months after transplantation. The 5-month duration of the posttransplantation study was based on noninvasive monitoring of the wall thickness of the grafts by MRI, performed as reported previously. By 5 months, it was apparent that the wall thickness in the EKO recipients and the mock donors was appreciably greater than that in the hAI/EKO group. In the previous study, the survival rate of the transplantation was 70%, and there was no difference between the EKO and hAI/EKO recipients. The recipient animal numbers given above were those that survived the surgery.

Transplantation Procedures

A segment of thoracic aorta (3 to 4 mm) was transplanted into an infrarenal position by microsurgical techniques as described. As in the previous study, the survival rate of the transplantation was approximately 70%, and there was no difference between the EKO and hAI/EKO recipients. The recipient animal numbers given above were those that survived the surgery.

Measurement of Plasma Lipids and ApoAI

HDL fractions were isolated from plasma samples by density-gradient ultracentrifugation. Total cholesterol and HDL-C were analyzed with commercial kits (Sigma Chemical Co). Human apoAI levels were measured by ELISA (Biodesign International) as described.

Histology, Immunohistochemistry, and Morphometry

Aortic samples were perfusion-fixed at 100 mm Hg with 4% paraformaldehyde in PBS. The nylon sutures identified graft termini. Thoracic aortas (pretransplant and mock donor group) or abdominal aortas containing the grafts (recipient groups) were removed en bloc with the posterior bony structures, fixed in 4% paraformaldehyde for 24 hours, decalcified overnight, sectioned transversely, and embedded in paraffin. Serial sections 5 μm thick were obtained and stained with combined Masson’s trichrome elastic stain as described previously.

For immunohistochemistry, sections were stained for α-actin, monocyte chemoattractant protein-1 (MCP-1), tissue factor, and acyl coenzyme A:cholesterol acyltransferase (ACAT) as previously described.
is described elsewhere.\textsuperscript{14} /H11005 (n = 5 to 9 animals per group, 7 sections per animal).

The mean lesion area of the serial sections was calculated for each individual graft from digitized images. The total area stained by the trichrome elastic stained serial sections as previously described.\textsuperscript{9}–\textsuperscript{14} and for CD68 (rat anti-mouse macrophages, Serotec; 2 
\textmu g/mL). The immunostaining protocol, including negative controls, is described elsewhere.\textsuperscript{14}

Morphometric measurements for lesion area were performed on the trichrome elastic–stained serial sections as previously described.\textsuperscript{9} The mean lesion area of the serial sections was calculated for each individual graft from digitized images. The total area stained by CD68 or SMC $\alpha$-actin was determined by a blinded investigator.

**Statistical Analysis**

Numerical data are expressed as either mean±SEM or mean±SD, as indicated, and were analyzed by ANOVA and t tests. Probability values of $P<0.05$ were considered significant.

**Results**

**Plasma Cholesterol and ApoAI Levels**

Non–HDL-C levels in the EKO mock donors, EKO recipients, and hAI/EKO were similar (Table). In contrast, there was a 2.4-fold elevation in the HDL-C levels in the hAI/EKO recipients compared with EKO mock donors and EKO recipients ($P<0.00001$), consistent with previous reports.\textsuperscript{2,3}

**Human ApoAI Expression Retarded Progression of Preexisting, Advanced Atherosclerotic Lesions**

After 6 months on WD, the mean lesion size in the thoracic aorta of donor EKO mice was $0.14\pm0.09$ mm\textsuperscript{2} (mean±SD).

\begin{table}[h]
\centering
\begin{tabular}{|l|c|c|c|}
\hline
 & HDL-C, mg/dL & Non–HDL-C, mg/dL & Human ApoAI, mg/dL \\
\hline
EKO donors† (n=14) & 32.3±7.9\textsuperscript{*} & 1070±437 & NA \\
Mock donors (n=6) & 27.2±7.8\textsuperscript{*} & 558±181 & NA \\
EKO recipients (n=7) & 26.3±4.2\textsuperscript{*} & 441±90 & NA \\
hAI/EKO recipients (n=9) & 63.8±11.1 & 514±120 & 130±45 \\
\hline
\end{tabular}
\caption{Mouse Plasma Cholesterol and ApoAI Levels} \\
\textsuperset{Values are mean±SD.} \\
\textsuperset{*P<0.00001 vs hAI/EKO recipients.} \\
\textsuperset{†On Western diet. All other groups were on chow diet.}
\end{table}

Five months after transplantation, aortic grafts were harvested from the EKO and hAI/EKO recipients. Morphometric analyses of thoracic aortic segments from the pretransplant and mock donor groups and of the grafts from the 2 recipient groups were performed. Representative photomicrographs from each group and a graphical summary of the numerical analyses are shown in Figure 2A and 2B, respectively. In Figure 2A, intimal lesions can be seen in all groups. Notably, all the lesions show evidence of being beyond the early foam-cell stage by having necrotic lipid cores (the cholesterol “clefts,” or clear structures). The intimal areas do not appear to be comparable among the groups, however, and the morphometric data (Figure 2B) confirm this impression; the mean lesion area in the grafts from the hAI/EKO recipients was significantly less than the lesion area measured in the EKO recipients or the mock donors (0.24±0.12 versus either 0.39±0.16 mm\textsuperscript{2}, $P<0.05$, or 0.37±0.05 mm\textsuperscript{2}, $P<0.05$, respectively). Compared with the pretransplant mean lesion area (0.14±0.09 mm\textsuperscript{2}), lesions in both the mock donors and the EKO recipients progressed significantly 5 months after transplantation. Importantly, these results could not be explained by nonspecific sequelae of the transplantation procedure, because there was no difference in the lesion size between the EKO recipient and mock donor groups. These results suggest that the elevation of HDL did not cause regression of preexisting, advanced lesions, but retarded further expansion of the neointima.

**Human ApoAI Expression Decreased Macrophage Content of Preexisting, Advanced Atherosclerotic Lesions**

The presence of macrophage-derived foam cells is a hallmark of the atherosclerotic lesion, and an increase in their content is thought to promote plaque instability. Therefore, in addition to the effect of HDL elevation on lesion size, we also determined the effects of HDL on lesion cellular composition. As shown in Figure 3A, immunostaining for the macrophage marker CD68 was considerably less in the lesion from the hAI/EKO recipient. In addition to a quantitative difference, there was also a qualitative difference in the localization of the macrophage staining, which was now confined to the base of the lesion and in the vicinity of the lipid core. The numerical results confirmed the visual assessment and are displayed in Figure 3B. Note the highly significant reduction (80\%, $P<0.003$) in the hAI/EKO group compared with the results in the other groups.
Similar results were also obtained by immunostaining for 3 other proteins expressed by lesion macrophages (Figure 4): MCP-1, a chemokine responsible for monocyte entry to vessel wall; ACAT, the enzyme responsible for intracellular cholesteryl ester synthesis and thus, foam-cell formation; and tissue factor, a protein with an important role in thrombosis subsequent to plaque rupture.

Human ApoAI Expression Increased SMC Content in Preexisting, Advanced Atherosclerotic Lesions

Plaque-stabilizing changes in lesions are thought to include reduced macrophage and increased SMC content. Therefore, we also determined the effect of HDL elevation on lesional SMC content by immunostaining for α-actin. As shown visually in Figure 5A and quantitatively in 5B, lesions in the grafts from hAI/EKO recipients had intimal staining for smooth muscle α-actin that was >3-fold greater than in any other group (P<0.003). The lesional α-actin contents in the mock donors and the EKO recipients were not significantly different. The SMC- and macrophage-related results, taken together, then, support the notion that HDL elevation may stabilize the plaque in a clinical setting.

Discussion

The present study used a novel mouse aortic transplantation model to investigate, for the first time, the effects of sustained HDL elevation on preestablished, advanced atherosclerotic lesions. Our results demonstrated that such an elevation retarded the further progression of lesions that had developed in the thoracic aortas of EKO mice fed WD for 6 months. In addition, the elevation in HDL was associated with intimal remodeling by dramatically decreasing macrophage-derived foam cells and increasing SMCs. Notably, these results could not be explained by either a difference in the total cholesterol levels or from sequelae of transplantation.

With aortic transplantation, the plasma lipoprotein environment of a lesion of any stage of development can be rapidly altered and the change sustained indefinitely. By transplanting lesion-containing thoracic aortic segments into EKO and hAI/EKO recipients, we were able to maintain the advanced lesions for 5 months in plasma environments that differed only in HDL-C levels (2.4-fold). The posttransplantation plasma lipoprotein levels in the recipients exactly mimicked the conditions of a previous study in which lesion formation was retarded in hAI/EKO compared with EKO mice. We were now able to ask whether this lipoprotein profile would beneficially affect preexisting, more advanced lesions. This question could not be addressed as effectively by previous methods, such as the infusion of HDL or apoAI or virus-mediated apoAI gene transfer, because of the inability in those studies to achieve a comparable degree or duration of increased HDL levels. Thus, we have been able to demonstrate that, even under the condition of hypercholesterolemia, advanced lesions can be remodeled beneficially after HDL elevation. These findings were not necessarily expected, given the prevailing view that only...
Limited changes can be induced in lesions after they advance beyond the early stages. 8

Compared with the EKO recipients, the smaller lesion size in the hAI/EKO recipients was attributable, at least in part, to decreased macrophage content. Many benefits of HDL appear to involve monocytes/macrophages directly or indirectly. For example, in HDL-mediated "reverse cholesterol transport," macrophages are presumed to be critical targets for cholesterol removal, particularly through the pathway mediated by ABCA1. 21 Consistent with this are the findings in Tangier disease, in which ABCA1 deficiency results in the absence of HDL from plasma and in macrophage cholesteryl ester accumulation throughout the reticuloendothelial system. In vitro, it has been directly shown that HDL stimulates cholesterol efflux from macrophages, and apoAI-mediated cholesterol efflux is greatly reduced in macrophages deficient in ABCA1. 22

Another way HDL may exert beneficial effects on macrophages is by preventing the formation of LDL-derived oxidized lipids or inactivating such lipids, as suggested principally by Fogelman and colleagues (eg, see Navab et al 23 and Van Lenten et al 24). The mediators of the antioxidant effects are thought to be HDL components, such as apoAI, apol, and lipid-modifying enzymes (eg, paraoxonase and platelet-activating factor acetylhydrolase). The expected result of elevated HDL would be, then, a decreased arterial content of reactive lipid species that promote inflammatory changes, including macrophage activation. For example, HDL suppresses the induction of MCP-1 in vascular wall cells by oxidized LDL. 25 HDL also downregulates tumor necrosis factor-α and interleukin-1-induced expression of vascular cell adhesion molecule-1 and other endothelial adhesion molecules important for monocyte recruitment. 26 Supporting clinical evidence for this mechanism is the observation that HDL isolated from patients with coronary heart disease had reduced anti-inflammatory activity. 21

Thus, the decreased macrophage content we observed could be a result of the effects of HDL on reverse cholesterol transport or oxidized lipids. It is also possible that after HDL-mediated efflux of cholesterol, the already resident macrophages disappeared as a result of necrosis, apoptosis, or egress. There is evidence in the literature to support the existence of these possibilities (eg, see References 2, 3, 27, 28, and 29), and our ongoing studies will focus on defining their relative roles and the specific effects of HDL on them.

In addition to the general decrease in lesion macrophage content in the hAI/EKO group, macrophages were particularly depleted in the subendothelial space. This could reflect decreased recruitment of monocytes into the vessel wall, preferential unloading of cholesterol from subendothelial macrophages, or migration of superficial macrophages into the vessel lumen. The loss of macrophages along a spatial gradient, as we have observed, bears a striking resemblance to the findings in classic studies in nonhuman primates. As summarized by Small, 30 when diet-sensitive monkeys, such as Macaca fascicularis, were fed a high-cholesterol diet for 18 months to induce lesions, then switched to low-cholesterol feeding, serial changes in the lesions also demonstrated a loss of the foam-cell “layers” in a spatial gradient from the subendothelium to the lipid core. This indicates that the remodeling of atherosclerotic lesions may be achieved by mechanisms and processes common among a number of mammalian species, thereby supporting the relevance of our findings to human atherosclerosis.

A surprising result in our study was the effect of HDL elevation on intimal SMC content. The increase in SMCs, in concert with decreased macrophages, should provide further stabilization. 31 The molecular basis for the increase can only be speculated upon at this time. HDL can affect SMC proliferation in vitro and may also affect SMC migration. Further studies will be needed to investigate these or other potential mechanisms.

Transplantation of advanced atherosclerotic lesions from EKO to hAI/EKO mice (exposing lesions to elevated HDL-C and non–HDL-C) resulted in remodeling but not regression. Similarly, infusion of a mutant form of human apoAI, apoAIMilano, thought to be a highly effective mediator of cholesterol efflux, resulted in a decrease in the content of macrophages and lipid but not in the size of advanced lesions in EKO mice. 33,34 In contrast, in our previous study, 9 transplantation of advanced lesions to wild-type mice (thereby exposing lesions to elevated HDL-C but decreased non–HDL-C) resulted in almost complete regression. This implies that either increased HDL level or function can only partially reverse the effect of hypercholesterolemia, or the
increased HDL level or function was insufficient and a further increase would overcome hypercholesterolemia and result in regression. Nonetheless, it is clear that in the present study, by shifting an advanced lesion to a higher-HDL environment, changes thought to be plaque-stabilizing resulted.

In conclusion, we have demonstrated that atherosclerotic lesions beyond the foam-cell stage can be favorably affected by the elevation of HDL. Furthermore, the results strongly suggest that we have established a valuable animal model, likely to be relevant to human lesion behavior, with which to pursue the mechanisms underlying lesion remodeling. It is our hope that this pursuit will not only increase the understanding of lesion remodeling at the fundamental level but also lead to the discovery of molecular targets that will serve as the bases for future therapeutic interventions.

Acknowledgments
This work was supported by NIH grants HL-61814 (Dr Fisher) and HL-33714 (Dr Breslow). Dr Choudhury is a Wellcome Trust International Fellow. We thank Veronica Gulle for technical assistance with histology and immunohistochemistry.

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_Circulation_. 2001;104:2447-2452
doi: 10.1161/hc4501.098952

_Circulation_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7322. Online ISSN: 1524-4539

The online version of this article, along with updated information and services, is located on the World Wide Web at:
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