Increased Cholesterol Efflux in Apolipoprotein AI (ApoAI)–Producing Macrophages as a Mechanism for Reduced Atherosclerosis in ApoAI(−/−) Mice

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Abstract—The concentration of apolipoprotein (apo) AI in the artery wall is thought to enhance cellular cholesterol efflux and protect against atherosclerosis. It has been shown that although macrophages do not make apoAI, they respond to it by increased cholesterol efflux. We hypothesized that macrophage production of apoAI would increase cholesterol efflux and reduce atherogenesis. In this study, we produced mice expressing human apoAI under the control of the macrophage-specific scavenger receptor-A promoter (mφ-AI). Human apoAI was detectable in the serum HDL fraction of mφ-AI transgenic mice at concentrations too low to affect serum cholesterol or HDL levels. Immunoblotting showed the presence of human apoAI in transgenic macrophage culture supernatants, mostly as lipoprotein-free protein, with a small component associated with HDL-like particles. Atherosclerosis studies using apoAI(−/−) mice transplanted with mφ-AI bone marrow showed that in the absence of macrophage-derived apoE, local expression of apoAI reduced diet-induced lesions in the proximal aorta. Additionally, mφ-AI macrophages showed a 40% increase in cholesterol efflux compared with control macrophages. These data support the hypothesis that apoAI production by macrophages in the artery wall is protective against atherosclerosis. This protection is likely mediated by increased cholesterol efflux and decreased foam cell formation in vivo. (Arterioscler Thromb Vasc Biol. 2001;21:1790-1795.)

Key Words: apoAI ■ transgenic mice ■ macrophage ■ foam cell

A polipoprotein AI (apoAI) is a major protein component of HDL. There is a well-established inverse correlation between HDL cholesterol and/or apoAI serum levels and the risk of coronary heart disease (CHD).1 Patients with low HDL or apoAI deficiency syndromes are at greater risk of developing premature atherosclerosis.2 Much information regarding the protective role of apoAI in CHD has been gained with mouse models of atherosclerosis. Transgenic mice that overexpress human apoAI show increased HDL cholesterol levels and reduced susceptibility to diet-induced atherosclerosis compared with matched controls.3,4 In addition, apoE-deficient [apoE(−/−)] mice, which develop hypercholesterolemia and are prone to spontaneous atherosclerosis,5,6 also show decreased lesion size when overexpressing human apoAI.7,8 These studies indicate that the protective effect of apoAI and the HDL system might overrule the atherogenic influences of hypercholesterolemia. Although it is not completely understood, the protective effect of apoAI may be related to its association with increased HDL production. HDL can be protective by such mechanisms as prevention of lipoprotein oxidation,9,10 decreased expression of adhesion molecules on vascular cells,11 and increased cholesterol efflux from cells that are involved in the initial development of the atherosclerotic plaque, such as macrophages.12

One of the initial events in atherogenesis is the migration and accumulation of macrophages in the artery wall.13 In a hypercholesterolemic environment, these arterial macrophages quickly become lipid-engorged foam cells by taking up cholesterol-rich lipoproteins through phagocytosis and receptor-mediated mechanisms that primarily involve the scavenger receptors SR-A and CD36,14–16 the LDL receptor (LDLR),17 and possibly the LDLR-related protein (LRP).18 Efficient cholesterol efflux from the macrophages is critical for the prevention of foam cell formation and subsequent protection against CHD. This is evident in the low HDL syndrome known as Tangier’s disease, which has recently been found to be a disorder of cholesterol efflux due to mutations in the ABC-1 transporter.19–21 Individuals with Tangier’s disease have very low levels of HDL cholesterol and apoAI and suffer from accelerated atherosclerosis. This supports the idea that cholesterol efflux from cells is essential for activation of the reverse cholesterol transport pathway and for cardiovascular health. Extracellular sources of apoAI have been shown to increase cholesterol efflux from macrophages in vitro,22 and the
Reverse Transcription–Polymerase Chain Reaction Analysis

Total RNA was extracted from various mouse tissues and peritoneal macrophages with the RNAeasy mini kit (Qiagen). Duplex polymerase chain reaction (PCR) was performed with the following primer sequences for h-apoAI: 5’-AAGGACCTGGCCACTGTGTA-3’ sense and 5’-TCTCCTCTGCACTTTCC-3’ antisense (301 bp). The expected PCR product for 18S RNA was 489 bp. PCR for SR-A was conducted with the following primer sequences: 5’-CACGTTCGTACAGCCTCC-3’ sense and 5’-ACCTGCCCTGTTCTCTTT-3’ antisense (631 bp). The cycling conditions for both apoAI and SR-A were initial denaturation for 10 minutes at 94°C followed by 35 cycles of 94°C for 1 minute, 60°C for 1 minute, and 72°C for 1 minute.

Determination of Cholesterol and Triglyceride Levels in Serum

Determination of serum cholesterol and triglyceride levels was done by colorimetric assay, as described previously.27

Lipoprotein Isolation and Preparation

Unless otherwise stated, fractionation of lipoproteins was done by raising the density of serum or culture supernatant to 1.019 g/mL for VLDL, 1.040 for LDL, and 1.210 for HDL. For h-apoAI Western blots, serum was raised to a density of 1.210 g/mL. Lipoprotein samples were desalted and concentrated with Microcon filters (Millipore) and stored at 4°C in the presence of protease inhibitors until time of analysis. Acetylated-LDL (acLDL) was prepared by repeated addition of acetic anhydride (Sigma) to LDL in a sodium acetate solution as described previously30 and dialyzed in 0.15 mol/L NaCl and 1 mmol/L EDTA at 4°C.

Western Blotting

After ultracentrifugation, lipoprotein samples were electrophoresed on a 4% to 20% polyacrylamide gel under reducing conditions. Proteins were transferred to nitrocellulose, and the membrane was blocked. h-ApoAI was detected with a monoclonal antibody (BioDesign) specific for apoAI. Bands were visualized by enhanced chemiluminescence (Amersham).

ELISA Assay for h-ApoAI

h-ApoAI was detected in serum samples and macrophage culture supernatants of transgenic mice by a specific ELISA. Ninety-six-well plates were coated with mouse anti-h-apoAI monoclonal antibody (BioDesign) overnight at 4°C. Plates were washed with PBST and blocked with 10% FBS/PBS for 2 hours at room temperature. Diluted standard and samples were placed on plates and incubated overnight at 4°C. After washes, peroxidase-conjugated anti-h-apoAI antibody (BioDesign) was incubated for 45 minutes with acetylated-D-glucosamine-3-sulfonic acid (Sigma) for ~20 minutes. Absorbance was measured with a microplate reader (Molecular Devices) at a wavelength of 405 nm.

Bone Marrow Transplantation

Bone marrow transplants were done according to the methods described previously and commonly used in our laboratory.29 Four weeks after transplant, mice were placed on a butterfat diet (ICN Biochemicals) containing 19.5% fat, 1.25% cholesterol, and 0.5% cholic acid for 12 to 16 weeks.

Quantification of Atherosclerosis in the Proximal Aorta

After 12 to 16 weeks on diet, mice were killed and hearts (with the aorta) were embedded in OCT and frozen in liquid nitrogen. Fifteen 10-μm cryosections (a total of 300 μm distally) were created starting at the region of the aortic sinus and were analyzed as described previously.30

1H-Cholesterol Efflux Studies in Macrophages

Cholesterol efflux in peritoneal macrophages was determined by a modified procedure from Lin et al.31 Peritoneal macrophages were

The presence of apoAI in the extracellular space is considered necessary for the activation of cholesterol efflux through the ABC-1 pathway.23 Because macrophages do not make apoAI, efficient efflux is dependent both on the ability of plasma apoAI to diffuse to the extracellular surface and on the local production of other proteins that may serve as acceptors, such as apoE.24,25 In the present study, we describe transgenic mice that carry the human apoAI cDNA driven by the macrophage-specific scavenger receptor SR-A promoter26 (referred to hereafter as mSR-A). We have used these mice to test the hypothesis that intracellular production of apoAI will increase cholesterol efflux and reduce atherosclerosis.

Methods

Animals

C57BL/6-TgN(APOAI)Rub (hereafter referred to as h-apoAI TgN) overexpressing human apoAI (h-apoAI) and B6.129P2-Apoa1tm1 unc mice were purchased from the Jackson Laboratory (Bar Harbor, Me). Unless otherwise specified, all mice were maintained in microisolator cages on a rodent chow diet (4.5% fat; Purina Mills, Harbor, Me). Unless otherwise stated, all mice were maintained in microisolator cages on a rodent chow diet (4.5% fat; Purina Mills Inc) and autoclaved, acidified water (pH 2.8) ad libitum. All experimental protocols were performed according to the guidelines of Vanderbilt University’s Animal Care Committee.

Generation and Identification of h-ApoAI Transgenic Mice

The human SR-A enhancer/promoter construct (pAl) was a generous gift of Dr Chris Glass.26 For the macrophage-specific expression of h-apoAI, an 868-bp cDNA of the h-apoAI gene was cloned into the EcoRI and BamHI sites 3’ of the SR-A promoter and 5’ of the human growth hormone splicing and polyadenylation sites. The 7-kb fragment (Figure 1A) was isolated by digestion with ClaI and NotI, injected into fertilized C57BL/6 oocytes, and reimplanted into C57BL/6 foster mothers. Mφ-AI transgenic mice were backcrossed to the apoE-deficient mice on the C57BL/6 background (hereafter referred to as mφ-AI/E−/−). Southern blot analysis was conducted on genomic DNA with the 828-bp h-apoAI cDNA fragment used as a probe.
Cells were incubated in efflux media without cholesterol acceptors at 0, 6, and 12 hours. Methyl-β-cyclodextrin (MBCD) treatment was used to remove cholesterol from the cells. Cholesterol efflux was calculated from the total counts in the supernatant and expressed as a percentage of the total counts in the cellular lysate.

Results

Detection of h-ApoAI Transgene Expression

Southern blot analyses of tail DNA for the presence of the h-apoAI transgene revealed 2 founder lines with different degrees of transgene integration (data not shown). First-generation founders were crossed with C57BL/6 mice to create colonies, and in all cases, the transgene was transmitted through the germline. For the purposes of the present study, all experiments were conducted with the highest-expressing founder line.

Reverse transcription (RT)-PCR analysis of h-apoAI expression in various mouse tissues and peritoneal macrophages showed a high expression level of apoAI in the macrophages of mḗ-AI transgenic mice (Figure 1B, upper panel). To a lesser extent, h-apoAI transgene expression was also evident in other macrophage-containing tissues such as lung and liver, with little to no expression in the kidney. The expression patterns of apoAI in the mḗ-AI transgenic mice were similar to the SR-A gene (Figure 1B, lower panel). Compared with our transgenic mice, h-apoAI transgene expression was previously described by Rubin et al.3,4

Detection of h-ApoAI Protein and Distribution on Lipoproteins in Transgenic Mouse Serum

Western blot analyses of fractionated serum showed that the distribution of h-apoAI in the HDL range, d=1.0400 to 1.21 g/mL (Figure 2). The levels of h-apoAI in sera of mḗ-AI mice were 20 to 30 µg/dL, as determined by specific ELISA. This is ≈0.02% of normal levels of apoAI, which are in the order of 120 µg/dL. The addition of this small amount of h-apoAI in plasma had no apparent effects on total serum cholesterol, triglyceride, or HDL levels (Table).

Peritoneal Macrophages of Transgenic Mice Express and Secrete h-ApoAI

Culture supernatants were assayed to determine whether h-apoAI is secreted from macrophages. h-ApoAI was measured by ELISA in 24-hour culture supernatants from mḗ-AI macrophages at a concentration of 250 ng/10⁶ cells. No h-apoAI was detected in C57BL/6 control macrophage supernatants. Macrophage secretion of h-apoAI was confirmed by Western blot of culture supernatants (Figure 3A). Immunoblot analyses of fractionated mḗ-AI macrophage culture supernatants revealed that the h-apoAI in culture media was mostly associated with the lipid-free, d>1.21g/mL fraction (Figure 3B). However, there were also significant amounts of transgenic apoAI detected in the HDL range, d=1.10 to 1.21 g/mL. These data demonstrate that h-apoAI produced by transgenic macrophages is secreted into the extracellular space and can form or associate with HDL-sized lipoproteins.

Production of h-ApoAI by Macrophages Protects Against Diet-Induced Atherosclerosis

Because apoAI is commonly thought of as an antiatherogenic molecule, we hypothesized that its delivery by macrophages to the artery wall would protect mice from atherosclerosis. To test this hypothesis, we transplanted mḗ-AI bone marrow into apoAI(−/−) recipients, thus ensuring that macrophages were the only source of apoAI. Four weeks after transplantation, the apoAI(−/−) mice were placed on a butterfat diet for 16 weeks. There were no differences in serum cholesterol or triglyceride levels between the 2 groups (data not shown). The h-apoAI concentration in the serum of mḗ-AI bone marrow recipients was 27.7±2.5 µg/dL. Because this value is similar to that found in transgenic mice, macrophages are...
most likely the major source of h-apoAI in md-AI transgenic mice. Atherosclerosis analyses revealed a 25% reduction in lesion area of md-AI recipient mice (2412±898 μm²; n = 13) compared with C57BL/6 recipient mice (3216±1200 μm²; n = 15). Although indicative of a beneficial effect, this difference did not reach statistical significance.

Macrophages also produce apoE, which has antiatherogenic properties; thus, we reasoned that the protective effects of macrophage apoAI might be more evident in the absence of macrophage-derived apoE. To test this, we transplanted apoE−/− mice with bone marrow from apoE−/− (n = 4), apoE−/−/md-AI (n = 6), and C57BL/6 (n = 7) mice. Our laboratory has shown previously that transplantation of apoE−/− marrow into C57BL/6 mice on a butterfat diet increases lesion area.29 Consistent with our previous report, apoAI−/− mice transplanted with apoE−/− bone marrow developed significantly more atherosclerosis than C57BL/6 recipient mice (Figure 4). As predicted by our hypothesis, apoAI−/− mice that received apoE−/−/md-AI bone marrow were significantly protected against atherosclerosis compared with apoE−/− recipients. The protection in the md-AI group was of the same degree (96% reduction in lesion area) as that observed in the C57BL/6 recipients (82% reduction in lesion area). These data suggest that in the absence of macrophage-derived apoE, an established atherogenic situation, macrophage production of apoAI is protective against diet-induced atherosclerosis.

**Production of h-ApoAI by Transgenic Macrophages Increases Cholesterol Efflux In Vitro**

To determine whether the reduced lesion area in apoAI−/− mice transplanted with apoE−/−/md-AI marrow was associated with an increase in macrophage reverse cholesterol transport, we assessed the ability of macrophage-secreted apoAI to increase cholesterol efflux from transgenic cells. Cholesterol loading with acLDL was performed to stimulate foam cell formation, as indicated by the foamy appearance of macrophages. At 6 hours, we observed an ~40% increase (P<0.01) in cholesterol efflux by cholesterol-loaded apoE−/−/md-AI macrophages compared with loaded apoE−/− macrophages (Figure 5A). This enhanced efflux was evident in the absence of exogenous extracellular acceptors. The higher efflux observed in wild-type C57BL/6 macrophages was likely a result of apoE synthesis.

To examine whether the inability of apoE−/−/md-AI macrophages to efflux at the same level as apoE+/+ wild-type macrophages was due to the higher concentration of acceptor protein in wild-type cells, efflux experiments were repeated in the presence or absence of cyclodextrin (MBCD). At low concentrations (<1 mmol/L), MBCD acts as a cholesterol shuttle between cells and high-capacity cholesterol acceptors such as apoAI.32 In the presence of MBCD, we observed increased cholesterol efflux from all groups of macrophages. Interestingly, cholesterol efflux in apoE−/−/md-AI macrophages increased to levels equal to that of wild-type E+/+ macrophages (Figure 5B), demonstrating that the MBCD-facilitated increase in efflux (represented as Δefflux, Figure 5B inset) was highest for the apoE−/−/md-AI macrophages.

**Discussion**

ApoAI is a major protein component of HDL. In the mouse and in humans, apoAI is made primarily by the liver and intestine. Patients and animal models with apoAI deficiency have highlighted the importance of this molecule in lipoprotein metabolism and its involvement in atherogenesis. In addition, the benefits of apoAI and efficient cholesterol efflux are evidenced by increased CHD in patients with Tangier’s disease.19,33 Interestingly, the absence of apoAI has also been demonstrated to be deleterious even in the presence of macrophage-derived apoE.34 The potential antiatherogenic role of apoAI has also been demonstrated in a reciprocal
manner. Transient overexpression of hepatic apoAI, by use of an adenoviral infection approach, resulted in decreased development of atherosclerosis in apoE-deficient mice. Similarly, transgenic overexpression of h-apoAI from the liver was also protective against arterial lesion formation in diet-induced atherosclerosis and in apoE-deficient mice. In all of these studies, the protective effect of apoAI overexpression or the harmful effects of the absence of apoAI were paralleled by an increase or decrease in HDL cholesterol, respectively. In the present study, we investigated whether local expression of apoAI in the artery wall at levels that do not affect plasma HDL concentrations would be beneficial in reducing atherosclerotic plaque formation.

In this report, we describe a transgenic mouse model of h-apoAI expression driven by the macrophage-specific SR-A promoter. h-ApoAI was detected in sera of transgenic mice and was found to be associated with the HDL (d=1.040 to 1.21 g/mL) fraction. Tissue culture of transgenic macrophages in lipoprotein-free media revealed that these cells did produce and secrete h-apoAI. Most of the macrophage-secreted apoAI was found in the lipid-free fraction (d>1.21 g/mL) of culture supernatants. This large distribution of lipid-free apoAI may be due in part to ultracentrifugation in a high salt concentration. Interestingly, we also found apoAI in the HDL fraction (d=1.100 to 1.210 g/mL). Although it is possible that the apoAI containing HDL was assembled in the extracellular space, these data are consistent with a previous study showing that macrophages can secrete HDL-sized particles. The production of macrophage-derived apoAI had no effect on total cholesterol, triglyceride, or HDL cholesterol in vivo (Table), as expected by the low level of h-apoAI in sera of transgenic mice. This contrasts with other models of transgenic apoAI expression in which plasma levels of h-apoAI and HDL were significantly increased compared with control animals.

Bone marrow transplantation experiments with apoAI-/- mice demonstrated a 25% reduction in atherosclerosis in mice that received m-/AI bone marrow compared with recipients of control marrow. Although notable, this difference was not statistically significant. This may be due to several factors. First, the level of apoAI produced by the macrophages in our transgenic mice may not be high enough to enhance cholesterol efflux above the levels set by apoE. Second, the apoAI-/- mice have been shown to develop small lesions similar to those seen in C57BL/6 mice, perhaps making it difficult to detect protective effects of macrophage-produced apoAI. Finally, the presence of apoE may mask any physiological function of apoAI in the models under study.

We have previously shown that transplantation of apoE-/- macrophages into normal C57BL/6 mice resulted in increased atherosclerotic lesion area after 12 weeks on a butterfat diet. Therefore, weexploited the usefulness of this system to test whether macrophage expression of apoAI could assume the physiological role of apoE. In agreement with our previously published data, mice that received apoE-/- bone marrow had increased atherosclerosis compared with the C57BL/6 marrow recipients. However, mice that received apoE-/-/m-/AI bone marrow had an astonishing 96% reduction in lesion area compared with apoE-/- recipients. These data suggest that small amounts of apoAI delivered to the artery wall and to the developing plaque are as protective against atherosclerosis as large amounts of macrophage-derived apoE.

Because lipid-free apoAI can enhance cellular cholesterol efflux, the possibility exists that the beneficial effect of an activated reverse cholesterol transport system may be evident even through more subtle modifications that do not affect plasma or serum HDL cholesterol levels. The reverse cholesterol transport model hypothesizes that in the presence of an appropriate cholesterol acceptor, such as apoAI, cholesterol-loaded cells are able to reduce their intracellular cholesterol stores by activating cholesterol efflux. When analyzed for the ability to efflux cholesterol, macrophages from apoE-/-/m-/AI mice showed significant increases relative to normal macrophages. In the presence of cholesterol loading and in the absence of exogenously added extracellular acceptors (such as HDL, apoAI, or apoE), the efflux of free cholesterol from apoAI-producing macrophages was higher after 6 and 12 hours that that from controls. Cyclodextrins have previously been shown to be catalysts of cholesterol efflux by acting as cholesterol shuttles and allowing for a more rapid equilibration of cholesterol between cells and extracellular acceptors. Treatment with low concentrations of cycloextrin facilitated a significantly greater increase in efflux from apoE-/-/m-/AI macrophages than from apoE-/- or wild-type apoE-/- macrophages. The greater cyclodextrin-dependent increase indicates that media conditioned by apoE-/-/m-/AI macrophages have a greater capacity for accepting cholesterol. This suggests that on secretion from macrophages, apoAI is not saturated with cholesterol. Alternatively, these data suggest that the amount of cholesterol in the cell membranes available for MBCD interaction may be different among the groups. This difference, however, would not account for the large increases in ∆efflux from apoE-/-/m-/AI macrophages, because total H-cholesterol counts in cell lysates were similar for all groups at the beginning of the efflux experiment. At any rate, these data support a model in which the production of apoAI may confer an autocrine or paracrine property to the macrophage, probably mediated by the accumulation of secreted apoAI outside the plasma membrane, where it activates cholesterol efflux. Such an antiatherogenic property related to increased cellular cholesterol efflux has been described for macrophage-derived apoE. ApoE and apoAI are thought to be antiatherogenic by several mechanisms. Because enhancement of cholesterol efflux is a shared function of apoAI and apoE, we believe that these experiments emphasize the protective effect of reverse cholesterol transport in the developing atherosclerotic plaque.

In conclusion, this is the first report of transgenic, macrophage-specific expression of h-apoAI in a mouse model. We have shown that macrophage-derived apoAI is present in serum at levels that do not affect HDL cholesterol and that this apoAI associates with the HDL fraction. We have also demonstrated that macrophage production of apoAI reduces the development of arterial lesions in one model of diet-induced atherosclerosis and that this reduction is likely due to enhanced cholesterol efflux. Therefore, the availability of apoAI in and around macrophages and macrophage-rich areas of the atherosclerotic plaque increases their ability to eliminate cholesterol from the vessel wall. The results from this study, and studies from our laboratory using retrovirus-mediated apoE expression, are the foundation for the future development of effective gene therapy.
methods using macrophages to target treatment directly to the artery wall, thereby activating local reverse cholesterol transport.

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