Testing the role of apoA-I, HDL, and cholesterol efflux in the atheroprotective action of low-level apoE expression

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Abstract **Low levels of transgenic mouse apolipoprotein E (apoE) suppress atherosclerosis in apoE knockout (apoE$^{-/-}$) mice without normalizing plasma cholesterol. To test whether this is due to facilitation of cholesterol efflux from the vessel wall, we produced apoA-I$^{-/-}$/apoE$^{-/-}$ mice with or without the transgene. Even without apoA-I and HDL, apoA-I$^{-/-}$/apoE$^{-/-}$ mice had the same amount of aorta cholesteryl ester as apoE$^{-/-}$ mice. Low apoE in the apoA-I$^{-/-}$/apoE$^{-/-}$ transgenic mice reduced aortic lesions by 70% versus their apoA-I$^{-/-}$/apoE$^{-/-}$ siblings. To define the free cholesterol (FC) efflux capacity of lipoproteins from the various genotypes, sera were assayed on macrophages expressing ATP-binding cassette transporter A1 (ABCA1). Surprisingly, ABCA1 FC efflux was twice as high to sera from the apoA-I$^{-/-}$/apoE$^{-/-}$ or apoE$^{-/-}$ mice compared with wild-type mice, and this activity correlated with serum apoA-IV. Immunodepletion of apoA-IV from apoA-I$^{-/-}$/apoE$^{-/-}$ serum abolished ABCA1 FC efflux, indicating that apoAI-V serves as a potent acceptor for FC efflux via ABCA1. With increasing apoE expression, apoA-IV and FC acceptor capacity decreased, indicating a reciprocal relationship between plasma apoE and apoA-IV. Low plasma apoE (1–3 × 10^{-8} M) suppresses atherosclerosis by as yet undefined mechanisms, not dependent on the presence of apoA-I or HDL or an increased capacity of serum acceptors for FC efflux.**—Thorngate, F. E., P. G. Yancey, G. Kellner-Weibel, L. L. Rudel, G. H. Rothblat, and D. L. Williams. **Testing the role of apoA-I, HDL, and cholesterol efflux in the atheroprotective action of low-level apoE expression.** ***J. Lipid Res.*** **2003.** 44: **2331–2338.**

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Atherogenic cholesteryl ester (CE)-rich lipoproteins promote the intimal accumulation of cholesterol-laden macrophage foam cells and the progressive development of atherosclerotic plaque (1). The prevention of atherosclerosis by apolipoprotein E (apoE) is generally attributed to the removal of plasma lipoprotein remnant particles; however, previous studies by our group suggest that apoE may have a role in preventing atherosclerosis in addition to its role in remnant clearance. In transgenic apoE-knockout (apoE$^{-/-}$) mice making mouse apoE specifically in the adrenal gland, we unexpectedly found that atherosclerosis was reduced by 80–95% in two transgenic lines expressing insufficient amounts of apoE to correct their hypercholesterolemia. This protection persists over the life of these animals, up to almost 2 years, with no detectable differences from apoE$^{-/-}$ siblings in their total plasma cholesterol or remnant lipoproteins (2).

Several groups have demonstrated that apoE expressed by macrophages in apoE$^{-/-}$ mice at levels too low to correct hypercholesterolemia significantly reduces atherosclerosis (3, 4). They suggest this protection is due to apoE production by macrophages in the arterial wall leading to increased efflux of cholesterol from arterial macrophages and thereby inhibiting foam cell development. The initial step in reverse cholesterol transport is hypothesized to be the release of free cholesterol (FC) and phospholipid from the plasma membrane of cells to acceptor particles such as HDL or lipid-poor apolipoproteins. Lipid-poor apoA-I stimulates release of cholesterol and phospholipid from macrophages via the ATP-binding cassette transporter A1 (ABCA1) (5, 6). ApoE is also an acceptor for FC and phospholipid released from macrophages by ABCA1 (7). Additionally, apoE has been

Abbreviations: ABCA1, ATP-binding cassette transporter A1; CE, cholesteryl ester; cpt-cAMP, 8-(4-chlorophenylthio)adenosine 3′,5′-cyclic monophosphate; FC, free cholesterol; LRP, low density lipoprotein receptor-related protein; PDGF, platelet-derived growth factor.

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reported to stimulate cholesterol release from macrophages to apoA-I-containing acceptors (8–10). ApoE also enhances HDL or apoA-I interaction with vascular wall cells and extracellular matrix (11), activities that could contribute to the atheroprotection due to low-level apoE expression. (12–14).

Overall, these findings from numerous laboratories suggest the hypothesis that low levels of systemic apoE may stimulate cholesterol efflux from arterial wall cells to apoA-I-containing acceptors, thereby suppressing atherosclerotic lesion development. To test this hypothesis, the 619 line of apoE transgenic mice on the apoE$^{-/-}$ background (2) was bred onto the apoA-I$^{-/-}$ background. Mice on the double knockout background with and without the adrenal apoE transgene were then compared for development of aortic lesions and for the capacities of serum lipoproteins to accept FC released from macrophages via the ABCA1 pathway as a measure of cholesterol efflux.

MATERIALS AND METHODS

Materials

[1,2-^{3}H]cholesterol (specific activity = 45 Ci/μmol) was from NEN Life Science Products Inc. Tissue culture flasks and plates were from Corning (Corning, NY) and Falcon (Lincoln, NJ). Minimum essential medium buffered with 25 mM HEPES (pH 7.4) [MEM-HEPES, RPMI 1640, phosphate-buffered saline, and sodium pyruvate were purchased from CellGro (Herndon, VA)]. The ACAT inhibitor, Pfizer CP-113,818, was a gift from Pfizer Inc. (Groton, CT). Other reagents were purchased from Sigma.

Mice

Mice were maintained in a specific pathogen-free environment on a 12 h light, 12 h dark cycle. They were provided with standard rodent chow containing 4.5% fat (PicoLab Rodent Diet 20, LabDiet, St. Louis, MO) and water ad libitum. The production of mice expressing transgenic mouse apoE specifically in the adrenal gland was described previously (2). C57BL/6J-Apoa1^{tm1Unc} mice (15) were purchased from the Jackson Laboratories (Bar Harbor, ME). At the State University of New York (Stony Brook, NY), the C57BL/6J-Apoa1^{tm1Unc} mice were bred with FVB/N mice (Taconic, Germantown, NY) to produce mice having an 8:1 FVB/N-C57BL/6 background, the same background as the apoE$^{-/-}$ transgenic mice. These mice were crossed with line 619 apoE$^{-/-}$ apoE transgenic mice and line 616 transgenic mice to generate apoA-I$^{-/-}$/apoE$^{-/-}$ transgenic mice and sibling apoA-I$^{-/-}$/apoE$^{-/-}$ mice. Line 616 apoE$^{-/-}$ transgenic mice produce levels of plasma apoE (3–10 μg/ml) sufficient to reduce plasma cholesterol to near the wild-type level (2). The genotypes of all offspring were determined by PCR analysis of genomic DNA as described for apoE$^{-/-}$ and the apoE transgene (2) or for apoA-I$^{-/-}$ (15). Housing and experimental procedures were approved by the State University of New York at Stony Brook Committee on Laboratory Animal Resources.

Measurement of cholesteryl ester deposition in the aorta

After 9 months to a year on the chow diet, mice were anesthetized with ketamine HCl (80 mg/kg) and xylazine (1.2 mg/kg) administered intraperitoneally. Blood was drawn by cardiac puncture, and plasma and aortas were prepared and assayed as described (2, 16).

Lipoprotein analyses

Mice were fasted for 6 h, anesthetized with inhaled isoflourane, and bled from the tail into heparin-coated capillaries. Plasma was obtained by centrifugation and was preserved with 1 mM EDTA, 50 μM butylated hydroxytoluene, 2 mM 5,5-dithiobis-(2-nitrobenzoic acid), 0.015% phenylmethylsulphonylflouride, and 0.05% sodium azide. Lipoprotein profiles were obtained from 200 μl of pooled plasmas of three to six male mice, between 12 and 20 weeks old, by chromatography on a 25 ml Superose 6 (Pharmacia) FPLC column as described (2). HDL fractions from the separations were pooled and total cholesterol determined using reagents from Wako Chemicals USA (Richmond, VA). Reported values of HDL cholesterol are the mean ± SE for three samples (each pooled from three mice) for each genotype.

Western blot

Plasma (1 μl) or FPLC column fractions (50 μl) were resolved on reducing 10% polyacrylamide-SDS gels. Samples were transferred electrophoretically to nitrocellulose and immunostained using a rabbit anti-mouse apoE antiserum (2), rabbit anti-mouse apoA-I (BioDesign International, Kennebunk, ME), and rabbit anti-rat apoA-IV (gifts from Gabriella Castro and Charles Bisgaier). The secondary antibody was a peroxidase-coupled anti-rabbit IgG (Amersham Biosciences). Bands were visualized using West Pico SuperSignal reagents (Pierce Biotechnology, Inc.). Gel bands were quantified with a BioRad Model GS-700 Imaging Densitometer and MultiAnalyst Software.

Cell culture

J774 mouse macrophages were cultured as described (6). THP-1 cells were routinely grown in suspension in RPMI media containing 25 μM HEPES, 10% fetal bovine serum, 50 μg/ml gentamicin, 1 mM sodium pyruvate, 50 μM β-mercaptoethanol, and 2.5 mg/ml glucose. Before each experiment, THP-1 monocytes were differentiated by plating the cells in 12 well tissue culture plates (400,000 cells/well) in growth media with the addition of 50 ng/ml phorbol 12-myristate 13-acetate for 3 days.

Preparation of serum fractions

All mice used to prepare serum for efflux assays were males between 12 and 25 weeks old. Mice were fasted for 6 h, anesthetized with ketamine and xylazine as above, bled by cardiac puncture, and the blood was allowed to clot overnight on ice. Sera were pooled from three to seven mice of the same genotype, aliquoted, and frozen at −80°C. ApoB was precipitated from serum by adding 100 μl of serum to 40 μl of 20% polyethylene glycol [Sigma P-2139 in 200 mM glycine (pH 10.0)] solution. This mixture was incubated at room temperature for 15 min. After this incubation, the solution was centrifuged at 10,500 rpm in a Marathon 16KM microfuge (Fisher Scientific) for 30 min to pellet the precipitate. The supernatant was removed from the pellet and diluted into tissue culture medium at the indicated concentrations.

Immunodepletion of apoA-IV from apoA-I$^{-/-}$/apoE$^{-/-}$ serum

Ten male apoA-I$^{-/-}$/apoE$^{-/-}$ mice were fasted and bled as above. The sera were pooled and split into two aliquots, one of which was immunodepleted and the other was treated identically without the addition of the anti-apoA-IV antiserum but with the addition of normal rabbit serum as a mock control. Two milliliters of anti-rat apoA-IV antiserum or normal rabbit serum were brought to pH 8.0 with 200 μl 1 M Tris HCl (pH 8.0). This was bound to 2 ml of Affi-gel protein A agarose beads (Bio-Rad), washed twice with ten column volumes of 100 mM Tris HCl (pH 8.0), and then twice with ten column volumes of 10 mM Tris HCl

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(pH 8.0). The washed beads with antibody bound were added to 2 ml of apoA-I$^{-/-}$/apoE$^{-/-}$ serum and rotated at 4°C for 2 h. As tested by Western blot, this resulted in an about 80% depletion of apoA-IV from the serum. The process was repeated, and no apoA-IV was detected in the serum (data not shown). The sera were frozen at −80°C until assayed.

Efflux assays

Radiolabeled cholesterol efflux assays were carried out as described previously (6). Briefly, J774 monolayers were washed with MEM-HEPES and then incubated for 24 h in media containing [1,2-^{3}H]cholesterol (2 μCi/well), 1% fetal bovine serum, and 2 μg/ml CP-113,818 ACAT inhibitor to ensure that all labeled cholesterol was present as FC. Following the 24 h labeling period, cells were washed and then incubated with 0.2% BSA in RPMI 1640 with or without 0.3 mM cpt-cAMP for 15 h to induce ABCA1. Some wells were washed with PBS, dried, and extracted with isopropyl alcohol to provide a baseline value for total [1,2-^{3}H]cholesterol content. Washed monolayers (with and without cpt-cAMP induction for each sample) were incubated for 4 h in the presence of 1% total serum or 1.4% of the HDL fractions from the various mouse sera. The percent of radiolabeled cholesterol released (percent efflux) was calculated as (cpm in medium at 4 h/cpm in cells at time 0) × 100. The percent efflux from cells not treated with cpt-cAMP was subtracted from the percent efflux from the treated cells to yield the percent efflux due to ABCA1.

For cholesterol mass efflux assays, after the differentiation period, THP-1 macrophages were enriched in unesterified cholesterol by incubation with 250 μg/ml FC-phospholipid dispersion (17) in RPMI medium supplemented as above, with 7% calf serum replacing the 10% FBS, plus 50 ng/ml PMA and 2 μg/ml CP113,818 for 24 h. The cells were then equilibrated for 18 h in RPMI medium containing 0.2% BSA (essentially fatty acid free; Sigma) in place of serum. Cells were harvested at this time to determine cholesterol content in the cells before the efflux period. Efflux media was applied for 8 h, after which media were removed and the cells were harvested as described above. Cholesterol content was determined by gas liquid chromatography, as previously described (18). Protein was determined using a modified Lowry assay (19).

Statistical analysis

Statistical analyses were performed using Prism version 3 software (GraphPad Software, San Diego, CA). Significance was determined using an unpaired *t*-test if the data distributions were Gaussian, or a Mann-Whitney U test if not. All values are presented as the mean ± SE.

RESULTS

To test the role of apoA-I in the protection afforded by low levels of apoE, the 619 line of apoE transgenic mice on the apoE$^{-/-}$ background was crossed onto the apoA-I$^{-/-}$ background. Male mice on the double knockout background were compared with and without the adrenal apoE transgene.

The absence of apoA-I expression had no measurable effect on total plasma cholesterol in the apoE$^{-/-}$ background with or without low-level apoE expression. Total plasma cholesterol for the apoE$^{-/-}$ mice was 462 ± 22 mg/dl, for the apoA-I$^{-/-}$/apoE$^{-/-}$ mice was 426 ± 40 mg/dl, and for the apoA-I$^{-/-}$/apoE$^{-/-}$ transgenic mice was 452 ± 46 mg/dl. Similarly, the absence of apoA-I had no effect on the gel exclusion chromatographic profile of lipoproteins in these mice. **Figure 1B–D** shows that the profile of VLDL remnants was the same in all three genetically altered mouse lines. ApoE deficiency leads to reduced HDL cholesterol levels (20), and this is seen here as well. In comparison to wild-type mice, apoE-deficient

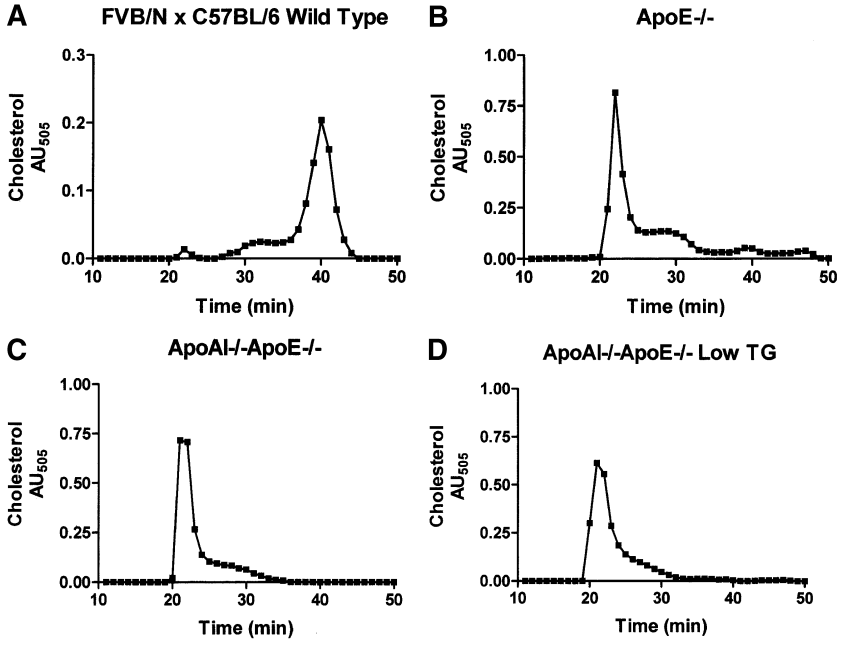

Fig. 1. Chromatographic profiles of plasma lipoproteins. Pooled plasma samples (200 μl of pooled plasmas of three to six male mice, between 12 and 20 weeks old) from the indicated genotypes were run on a Superose 6 column, and cholesterol was measured in each fraction as described in Materials and Methods. Fractions 19–23 contain VLDL and remnants, fractions 24–34 contain IDL and LDL, and fractions 36–42 contain HDL.

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mice had about one third as much HDL cholesterol (27 ± 8 mg/dl vs. 80 ± 4 mg/dl) and this was not changed substantially by low-level transgenic expression of apoE in the apoE$^{-/-}$ mice (29.8 ± 14 mg/dl). HDL cholesterol was not detectable in the apoA-I$^{-/-}$/apoE$^{-/-}$ mice and was barely detectable in only one of three pooled samples in the apoA-I$^{-/-}$/apoE$^{-/-}$ mice with low-level expression of the apoE transgene (1.0 mg/dl). Thus, the absence of apoA-I essentially eliminated HDL cholesterol in the apoE-deficient mice with or without the transgene.

The absence of apoA-I did not affect the deposition of CE in the aorta of apoE$^{-/-}$ mice relative to apoE$^{-/-}$ mice with apoA-I (**Fig. 2**). Both groups had the same range of aortic CE with means of 37 ± 5 μg CE/mg aorta protein for apoE$^{-/-}$ mice versus 36 ± 8 μg CE/mg aorta protein for apoA-I$^{-/-}$/apoE$^{-/-}$ mice. In contrast, low-level transgenic expression of apoE in apoA-I$^{-/-}$/apoE$^{-/-}$ double knockout mice was still sufficient to protect the mice. These mice had 12 ± 2 μg CE/mg aorta protein ($P < 0.001$ vs. their apoA-I$^{-/-}$/apoE$^{-/-}$ siblings by Mann-Whitney U test). Thus, even without apoA-I and HDL to mediate reverse cholesterol transport, low-level apoE provides protection against lesion development in 9- to 12-month-old mice.

In order to test whether the apoE$^{-/-}$ or apoA-I$^{-/-}$/apoE$^{-/-}$ mice, with or without the apoE transgene, have a reduced capacity for serum lipoproteins to serve as acceptors for cholesterol efflux from cells, we tested these sera with J774 macrophages in which the ABCA1 transporter was up-regulated by treatment with cpt-cAMP (6). Parallel assays were performed with cells not stimulated with cpt-cAMP. The difference in FC efflux with and without cpt-cAMP treatment represents the contribution of the ABCA1 pathway (6). In these assays, J774 cells labeled with [^{3}H]cholesterol were incubated with either 1% serum or with an equivalent amount of serum from which the apoB-containing lipoproteins were removed by polyethylene glycol precipitation. As shown in **Fig. 3A**, sera from apoE$^{-/-}$ mice promoted a greater percentage of FC efflux compared with sera from wild-type mice. This increased FC acceptor capacity was also seen with sera from apoA-I$^{-/-}$/apoE$^{-/-}$ mice. Interestingly, sera from apoE$^{-/-}$ or apoA-I$^{-/-}$/apoE$^{-/-}$ mice expressing transgenic apoE at either a low or a higher level reduced FC efflux back to or near the value with sera from wild-type mice. Similar results

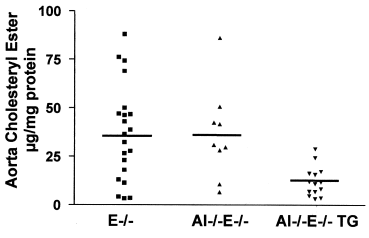

Fig. 2. Aortic cholesteryl ester (CE) accumulation. Aortic CE was measured by gas liquid chromatography in individual aortas of the indicated genotypes. The mean value for the apolipoprotein A-I (apoA-I)$^{-/-}$/apoE$^{-/-}$ mice expressing the line 619 apoE transgene (low apoE) differs from the values for the other two genotypes ($P < 0.001$ by Mann-Whitney U test).

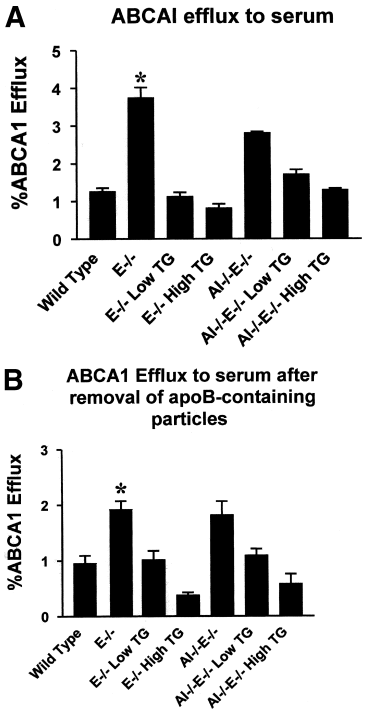

Fig. 3. ATP-binding cassette transporter A1 (ABCA1)-mediated free cholesterol (FC) efflux. J774 macrophages labeled with [^{3}H]cholesterol and treated with cpt-cAMP to induce ABCA1 were used as donor cells for FC efflux to equivalent volumes of serum pooled from mice of the indicated genotypes. A: Shows FC efflux to diluted sera and B shows efflux to diluted sera after removal of apoB-containing lipoproteins by polyethylene glycol precipitation. * E$^{-/-}$ differs ($P < 0.0005$) from wild-type, E$^{-/-}$ low TG, and E$^{-/-}$ high TG. Additionally, in A the following comparisons were made: AIE versus AIE low TG, $P = 0.013$; AIE versus AIE high TG, $P < 0.001$; AIE low TG versus AIE high TG, $P = 0.001$. In B, AIE versus AIE low TG, $P = 0.04$; AIE versus AIE high TG $P = 0.04$; AIE low TG versus AIE high TG, $P < 0.001$.

were obtained with sera from which apoB-containing lipoproteins were removed (Fig. 3B). One concern in these experiments is that ABCA1 levels in the J774 cells might change during the assay period due to exposure to different sera. ApoA-I and apoA-II have been reported to stabilize ABCA1 (21). To control for this, we repeated the efflux assays for 2 h instead of 4 h and used Western blotting to monitor ABCA1 levels. The 2 h assays showed the same results as in Fig. 3, and the Western blots showed little difference in ABCA1 levels with the various sera (data not shown).

ABCA1 will mediate cholesterol and phospholipid efflux to any lipid-poor exchangeable apolipoprotein (6). A potential candidate is apoA-IV, which shares many properties with apoA-I. As shown in the Western blot in **Fig. 4A**, the amount of apoA-IV in equal volumes of the various sera was increased in apoE$^{-/-}$ and apoA-I$^{-/-}$/apoE$^{-/-}$ mice compared with wild-type mice. ApoA-IV levels were decreased upon transgenic expression of apoE in either background. When apoA-IV was quantified by densitometry and plotted versus the ABCA1 FC acceptor capacity of the sera (Fig. 4B), a strong positive correlation ($r^2 = 0.771$) was seen.

These data are suggestive that apoA-IV is responsible, at

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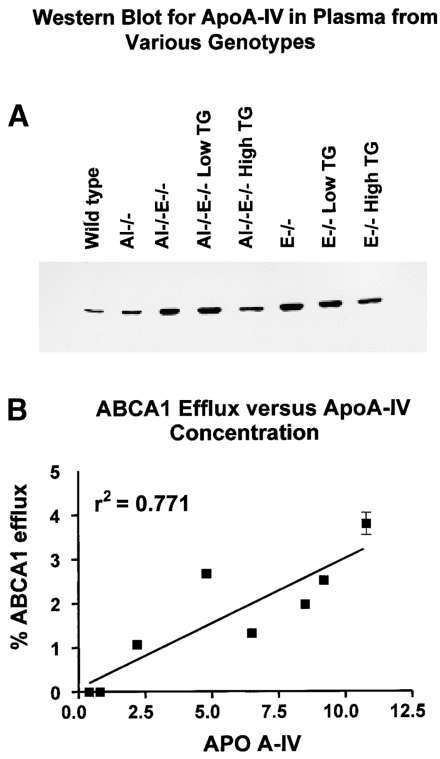

Fig. 4. Relationship between ABCA1-mediated FC efflux and serum apoA-IV concentration. A: Shows a Western blot for apoA-IV in equivalent volumes of pooled sera from the indicated genotypes. B: Shows the correlation between the apoA-IV concentrations determined by densitometry and the ABCA1-mediated FC efflux activity of the sera.

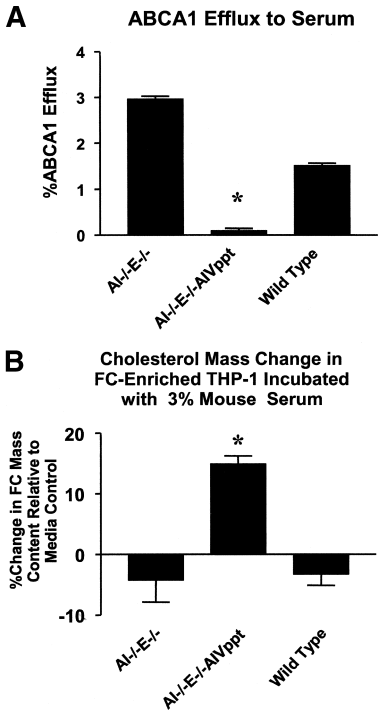

Fig. 5. Effect of apoA-IV depletion on FC efflux. Serum from apoA-I$^{-/-}$/apoE$^{-/-}$ mice was mock-treated or immunoabsorbed with rat anti-apoA-IV antibody as described in Materials and Methods. After centrifugation, volumes of apoA-I$^{-/-}$/apoE$^{-/-}$ sera containing equivalent amounts of total cholesterol were used as acceptors in FC efflux assays in comparison to an equivalent volume of serum from wild-type mice. A: Shows ABCA1-mediated FC efflux from J774 cells treated with a cpt-cAMP. B: Shows the change in total cell cholesterol mass when the sera were incubated with differentiated, cholesterol-loaded human THP-1 cells. * Differs ($P <$ 0.005) from AI$^{-/-}$ E$^{-/-}$ and wild-type.

least in part, for the enhanced ABCA1-mediated efflux in the apoE$^{-/-}$ and apoA-I$^{-/-}$/apoE$^{-/-}$ sera. To test this directly, apoA-IV was removed from apoA-I$^{-/-}$/apoE$^{-/-}$ serum by immunoabsorption with anti-rat apoA-IV and then tested for FC efflux activity on J774 cells. To account for dilution and losses during the immunoabsorption procedure, equivalent amounts of lipoproteins (based on cholesterol measurements) for the immunodepleted and mock-treated sera were added to the efflux assay. As compared with the mock-treated apoA-I$^{-/-}$/apoE$^{-/-}$ serum, the apoA-IV-depleted serum showed a 90% reduction in ABCA1 acceptor capacity (**Fig. 5A**). To further test the importance of apoA-IV as a FC acceptor in the serum of apoA-I$^{-/-}$/apoE$^{-/-}$ mice, we determined the change in cellular cholesterol mass mediated by the immunodepleted and mock-treated sera. For this purpose, human THP-1 monocytes were induced to differentiate and were loaded with FC as described in Materials and Methods. As shown in Fig. 5B, cholesterol mass decreased in THP-1 macrophages incubated with wild-type or apoA-I$^{-/-}$/apoE$^{-/-}$ mock-treated sera, but actually increased in cells incubated with apoA-IV-depleted serum. The increase in cholesterol mass with the immunodepleted serum may reflect the uptake of cholesterol-rich remnant particles that is not counterbalanced by ABCA1-mediated FC efflux to apoA-IV, but the mechanism of this increase has not been determined. These results demonstrate that apoA-IV is responsible for the increase in ABCA1-mediated FC efflux capacity in the serum of apoA-I$^{-/-}$/apoE$^{-/-}$ mice and indicate that apoA-IV can promote net cholesterol clearance from cholesterol-loaded THP-1 macrophages.

The distribution of apoA-IV among plasma lipoproteins was determined by gel exclusion chromatography followed by Western blotting. In plasma from wild-type mice (**Fig. 6A**), apoA-IV was present in two regions of the profile: fractions 38–40, the major HDL peak coincident with apoA-I, and fractions 44–48, which represents smaller particles or lipid-poor apoA-IV (serum albumin peaks in fraction 43). In apoE$^{-/-}$ plasma (Fig. 6B), apoA-IV accumulated in both the HDL and the smaller fraction and appeared in large remnant particles (fraction 20). In the plasma of apoA-I$^{-/-}$/apoE$^{-/-}$ mice (Fig. 6C), apoA-IV accumulated in large and smaller remnant particles (fractions 20–28) as well as in HDL-sized and smaller particles. With low expression of the apoE transgene in the apoA-I$^{-/-}$/apoE$^{-/-}$ mice, the apoA-IV distribution was shifted toward smaller remnants (fractions 22–32) as well as occurring in the HDL and smaller particles (Fig. 6D). With higher apoE expression (Fig. 6E), apoA-IV was found only in HDL and the smaller fractions. Note that in the transgenic mice, apoE was found exclusively in the remnant particles and not in HDL (Fig. 6D, E).

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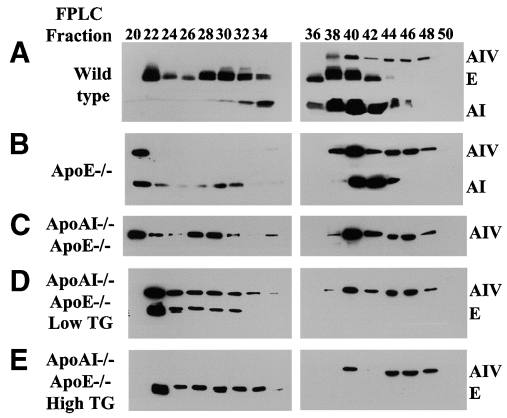

Fig. 6. Distribution of apoA-IV among plasma lipoproteins plasmas from the indicated genotypes (A–E) (200 μl of pooled plasmas of three to six male mice, between 12 and 20 weeks old) were resolved by chromatography on a Superose 6 column, and the indicated column fractions were run on 10% polyacrylamide-SDS gels and transferred to nitrocellulose. The blots were probed simultaneously with rabbit antisera against apoA-IV, apoE, and apoA-I, followed by peroxidase-coupled anti-rabbit IgG . Secondary antibody was visualized by enhanced chemiluminescence. Bands corresponding to apoA-IV, apoE, and apoA-I are indicated to the right of each profile.

DISCUSSION

These experiments tested the hypothesis that the protection against atherosclerosis afforded by low-level expression of apoE is mediated by enhanced cholesterol efflux from vascular wall cells to apoA-I or HDL. The results showed that low-level apoE (<1 μg/ml) suppressed lesion development in 9- to 12-month-old mice in the absence of apoA-I and apoA-I-containing HDL. In fact, HDL cholesterol in the apoA-I$^{-/-}$/apoE$^{-/-}$mice was not detectable and was unchanged by apoE expression. Since ABCA1 is believed to play a major role in cholesterol removal from macrophages of vascular lesions (22, 23), we examined the capacity of sera from these mice to serve as acceptors for this cholesterol efflux pathway. The results showed, surprisingly, that the ABCA1 acceptor capacity of serum was greatly increased in apoE$^{-/-}$ and apoA-I$^{-/-}$/apoE$^{-/-}$ mice, and this was reversed by transgenic expression of apoE. Taken together, these data demonstrate that low level apoE expression protects against atherosclerosis, but not by enhancing FC efflux via the ABCA1 pathway or by facilitating reverse cholesterol transport via apoA-I or apoA-I-containing HDL. The absence of HDL cholesterol in the apoA-I$^{-/-}$/apoE$^{-/-}$ mice suggests that other HDL-dependent FC efflux pathways also do not play a role in mediating the atheroprotective effects of low levels of apoE. In fact, the relative ability of serum to promote cholesterol efflux appears unassociated with the extent of atherosclerosis in these lines of mice.

In addition to its role in reverse cholesterol transport, apoA-I and HDL have a variety of other effects that may contribute to the antiatherogenic activity of HDL. Included among these are the ability to protect LDL from oxidation, the removal of oxidized lipids from LDL, antiinflammatory actions, inhibition of cytokine-induced vascular adhesion molecule expression, and regulation of vascular tone via activation of endothelial nitric oxide synthase (24–26). The present results indicate that low-level apoE does not suppress lesion development by acting via these apoA-I-dependent pathways.

The present results, as well as a previous study (2), show that low levels of systemic apoE suppress lesion development in the absence of macrophage apoE expression. Thus, although macrophage expression of apoE (3) or transgenic apoE expression in vascular wall cells (27) provides protection against atherosclerotic lesion development, our results indicate that the presence of systemic apoE, and not the cell of origin or whether apoE is made locally, is the key factor suppressing lesion development. Western blot analysis showed that transgenic apoE was present exclusively on remnant particles (Fig. 6D). The concentration of plasma apoE in the 619 line of mice is ~0.5–1 μg/ml or 1.5–3 × 10^{-8} M. We estimate the concentration of remnant lipoproteins as ~2 × 10^{-6} M, suggesting that only about 1–2% of the remnant particles could contain an apoE molecule. [Estimates of apoB-48 or remnant particle concentrations are based on measurements of core lipids (CE and triglycerides) which were assumed to account for 76% of remnant particle mass. ApoB-48 was assumed to be 8% of remnant particle mass.] Whether the apoE-containing remnants or a much smaller fraction of unassociated apoE is the active agent, these values strongly suggest that apoE is acting in a hormonal or cytokine-like manner to suppress lesion development and not through its ability to mediate remnant clearance.

Previous studies showed that apoE has a variety of hormonal or cytokine-like effects in steroidogenic cells (28, 29), platelets (30), and lymphocytes (31). More recently, Hui and colleagues showed that apoE inhibits the platelet-derived growth factor (PDGF) stimulation of smooth muscle cell proliferation and migration (32). The inhibition of PDGF-stimulated cell migration appears to be mediated via suppression of signal transduction through the LDL receptor-related protein (LRP). The LRP and other members of the LDL receptor family participate in signal transduction pathways in the brain and other cells (33). The LRP is tyrosine phosphorylated on its cytoplasmic tail via Src family kinases, leading to recruitment of downstream signaling components (34–36). Tyrosine phosphorylation of LRP is stimulated by PDGF (35, 36), and this activity is inhibited by apoE (35). These findings suggest a mechanism by which apoE might inhibit atherogenesis in the arterial wall via suppression of vascular smooth muscle cell migration and proliferation (35–37). In support of this hypothesis, inactivation of the LRP gene in vascular smooth muscle cells caused activation of PDGF signaling, smooth muscle cell proliferation, and enhanced aortic lesion development in LDL receptor-deficient mice (38). Further studies will be needed to determine whether apoE suppression of this pathway in vivo accounts, at least in part, for the suppression of atherosclerotic lesion development observed in the present study.

In the present study, apoE$^{-/-}$ mice with a mixed FVB/N-C57Bl/6 (8:1) background were used rather than the pure

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C57Bl/6 background that is more commonly used for atherosclerosis studies. The mixed background was used to overcome the poor reproductive performance of the apoE$^{-/-}$/apoA-I$^{-/-}$ mice with the C57Bl/6 background (data not shown). Comparison of the apoE$^{-/-}$ mouse with the FVB/N-C57Bl/6 (8:1) background with the apoE$^{-/-}$ mouse on the C57Bl/6 did not show a difference in aortic CE accumulation after 9–12 months on chow. For example, the apoE$^{-/-}$ on the mixed background had 37 ± 5 μg CE/mg aorta protein; the apoE$^{-/-}$ C57Bl/6 had 37.9 ± 1 μg CE/mg aorta protein (MannWhitney U test, $P = 0.86$). Total plasma cholesterol for the two apoE$^{-/-}$ backgrounds were FVB/N-C57Bl/6 (8:1) 462 ± 22 mg/dl; C57Bl/6 555 ± 57 mg/dl (*t*-test, $P = 0.59$). This result differs from a previous study comparing the pure C57Bl/6 with the pure FVB/N background (39) that showed the C57 to develop more atherosclerosis than the FVB. That comparison was at only 4 months of age compared with the 9–12 months in our comparison. Whether this difference with the previous study is due to the age of the mice or the mixed background in our mice is not known.

A surprising result in this study is the observation that a potent FC acceptor for ABCA1-mediated efflux accumulated in the serum of apoE$^{-/-}$ and apoA-I$^{-/-}$/apoE$^{-/-}$ mice. Western blot analysis showed that that the apoA-IV concentration correlated with the FC efflux activity. Immunoabsorption with anti-apoA-IV removed the acceptor activity, confirming its identity. Immunoabsorption of the apoA-IV also eliminated the ability of serum from apoA-I$^{-/-}$/apoE$^{-/-}$ mice to promote net cholesterol removal from cholesterol-loaded human THP-1 macrophages. Thus, apoA-IV is a potent acceptor for ABCA1-mediated FC efflux from macrophages. We anticipate that apoA-IV in the smaller lipid-poor fraction is the primary acceptor for ABCA1-mediated FC efflux in apoE$^{-/-}$ and apoA-I$^{-/-}$/apoE$^{-/-}$ sera since ABCA1 interacts preferentially with lipid-poor apolipoproteins (5, 6, 22). This has been shown to be the case in serum from apoA-IV transgenic mice in which the acceptor activity for ABCA1, as assayed in the present study, was found in lipoprotein-deficient serum but not in HDL (40). Although apoA-IV is a potent acceptor for ABCA1-mediated FC efflux, it does not appear to explain the atheroprotective effect of low-level apoE expression since apoA-IV concentrations were decreased upon transgenic apoE expression.

In all the genotypes studied, apoA-IV was present in HDL-sized particles and in a smaller, apparently lipid-poor, fraction as noted previously in wild-type and apoA-IV transgenic mice (40–42). Additionally, in the apoE$^{-/-}$ mice, apoA-IV accumulated in the largest VLDL remnants (Fig. 6). In the absence of apoA-I, apoA-IV was in the largest remnants but also accumulated throughout the smaller sized VLDL remnant particles. Low-level apoE expression shifted the apoA-IV from the largest to the smaller remnants, whereas with higher apoE expression, apoA-IV was completely absent from remnant particles. The interrelationship among these serum pools of apoA-IV is not understood, although the relatively weak lipid affinity of apoA-IV (43) may indicate that apoA-IV is in rapid equilibration among these pools and is displaced by apoE from remnant particles. The mechanisms by which apoA-IV accumulates in sera of apoE$^{-/-}$ and apoA-I$^{-/-}$/apoE$^{-/-}$ mice and the reduction in apoA-IV concentration upon low-level apoE expression are unknown. One possibility is that apoA-IV is normally cleared from the circulation via chylomicron remnant particles. Reduction of remnant clearance in the apoE$^{-/-}$ mice may permit apoA-IV accumulation and equilibration with the HDL and lipid-poor fractions. Alternatively, apoE may somehow influence the apoA-IV production rate. Additional studies will be needed to test these possibilities.

Transgenic over-expression of apoA-IV in C57Bl/6J mice reduced the size of aortic lesions when mice were fed a high-fat diet (41, 42). This was also the case in apoE$^{-/-}$ mice on a chow diet (42). One mechanism for this reduction in lesion size may be the ability of apoA-IV to promote FC efflux from vascular wall macrophages via the ABCA1 pathway. The studies reported here as well as those of Fournier et al. (40) are consistent with the idea that a primary physiological role of apoA-IV is as an acceptor for FC efflux from peripheral cells, including cells of the vascular wall. Additionally, the studies of Roheim and colleagues (44–46) demonstrated that apoA-IV is highly enriched in interstitial fluid lipoproteins relative to plasma HDL, findings that also support the idea that apoA-IV functions as a cholesterol acceptor at the first steps of reverse cholesterol transport. Additional studies to evaluate the role of physiological concentrations of apoA-IV on reverse cholesterol transport in vivo are clearly warranted.

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