Determination of the lower threshold of apolipoprotein E resulting in remnant lipoprotein clearance[1]

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Abstract Apolipoprotein E (apoE) is the ligand for receptor-mediated clearance of remnant lipoproteins. ApoE at concentrations only 10% of normal, achieved through transplantation of wild-type marrow into apoE(−/−) mice, is sufficient for the maintenance of normal serum lipid and lipoprotein levels. The goal of the present study was to identify the minimal concentration of serum apoE still affecting cholesterol levels, and to determine whether any effects on remnant clearance below this level of apoE were detectable. ApoE(+/+) marrow was mixed with apoE(−/−) marrow in proportions of 1, 5, 10, and 25% to make chimeric mice with serum levels of apoE ranging from 0.005 to 0.46 mg/dl. Analysis of serum cholesterol and apoE levels demonstrated a positive correlation between apoE levels and cholesterol reduction ($r = 0.83$), with levels of 0.04 mg/dl representing the functional threshold level. There were no differences in lipoprotein profiles and clearance between apoE(−/−) mice and mice with serum apoE of less than 0.04 mg/dl, as assessed by FPLC, non-denaturing gel electrophoresis, and turnover studies. However, electron microscopy of negative stains showed fewer lipoprotein particles with a diameter of <30 nm in the serum of these mice compared to apoE(−/−) mice. These data demonstrate that the threshold of serum apoE resulting in cholesterol reduction is 0.04 mg/dl, and indicate that apoE below this level affects lipoprotein size distribution possibly by accelerating the clearance of smaller remnants.—Hasty, A. H., M. F. Linton, L. L. Swift, and S. Fazio. **Determination of the lower threshold of apolipoprotein E resulting in remnant lipoprotein clearance.** *J. Lipid Res.* **1999.** 40: **1529–1538.**

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Apolipoprotein E (apoE) is a plasma glycoprotein responsible for the receptor-mediated clearance of remnant lipoproteins from the circulation (1–3). It is recognized by both the low density lipoprotein receptor (LDLR) and the LDLR related protein (LRP) (1), and also binds to heparan sulfate proteoglycans (HSPGs) (4, 5). Mutations in the apoE gene resulting in receptor-binding defective apoE or the complete absence of apoE lead to the development of type III hyperlipidemia (type III HLP) (6), which is characterized by an accumulation of cholesterol

and triglycerides (7–9), as well as by early atherosclerosis (9). An animal model of apoE deficiency was created by targeted disruption of the apoE gene in mice (10–12). These mice have characteristics similar to type III HLP patients, including hypercholesterolemia and spontaneous atherosclerotic lesion development (11–13).

ApoE in both humans and mice is present in serum at a concentration of 5–8 mg/dl (14, 15). The exact levels of apoE in serum needed to maintain cholesterol homeostasis are unknown, but normal apoE concentrations are well above the minimum level. Several lines of evidence point to the over-abundance of apoE in serum. Individuals and mice with only one apoE allele have no detectable abnormalities in their cholesterol metabolism although they express only half of normal apoE levels (8, 11, 12, 16). Additionally, serum apoE levels in apoE(−/−) mice after transplantation with apoE(+/+) marrow are only 10% of normal, yet produce a near normalization of serum cholesterol levels (17–20). Furthermore, transplantation of apoE(−/−) mice with apoE heterozygous marrow (+/−) results in serum apoE concentrations of only 5% of wild-type levels, which produces a significant reduction in plasma cholesterol levels in the order of 50% (17).

The goal of the present study was to create mice with levels of apoE below 5% of normal for the purposes of defining the threshold below which its effects on serum cholesterol are no longer apparent. To produce mice with varying low levels of apoE in serum, we transplanted apoE(−/−) mice with apoE(−/−) marrow mixed with

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apoE(+/+) marrow in decreasing proportions (1:4, 1:10, 1:20, and 1:100).

Here we demonstrate that the concentration of mouse apoE in serum necessary for significant reductions in serum cholesterol levels is 0.04 mg/dl, and that the cholesterol reduction is highly correlated with the log of serum apoE levels ($r = 0.83$). Although no differences in the clearance of remnant lipoproteins were detected in mice expressing low levels of apoE compared to apoE(−/−) mice, a significant reduction in smaller remnants was evident by electron microscopic analysis. These data support the concept of a narrow range of serum apoE concentrations that is limiting for detectable cholesterol changes. In this range, apoE shows a dose-dependent effect on remnant removal, from complete clearance at a level of 0.2 mg/dl to lack of any detectable clearance at a level of 0.03 mg/dl.

MATERIALS AND METHODS

Animal procedures

ApoE(−/−) mice used in transplant experiments were backcrossed at least 10 times into the C57BL/6 background. Mice were maintained in microisolator cages on a rodent chow diet (PMI No. 5010, St. Louis, MO) and were given autoclaved acidified water (pH 2.8). One week prior to and 2 weeks after bone marrow transplantation (BMT), mice were given antibiotic water consisting of 100 mg/l neomycin (Sigma) and 10 mg/l polymyxin B sulfate (Sigma), pH 2.0. Animal care and experimental procedures were performed according to the regulations of Vanderbilt University's Animal Care Committee.

ApoE purification and quantitation

ApoE was purified from 10 ml of pooled serum from C57BL/6 mice according to standard procedures (21). Briefly, a d < 1.040 lipoprotein cut was collected from serum and dialyzed against 5 mm NH_4HCO_3. After dialysis, the sample was lyophilized and resuspended in 0.1 m NH_4HCO_3. The sample was then delipidated with a series of ethanol:ethyl ether mixtures and resuspended in a guanidine HCl buffer (6 m guanidine HCl, 0.1 m Tris, 0.01% EDTA, pH 7.4). ApoE was separated from apoB by gel filtration chromatography on a Sepharose-200 column (1.6×200 cm) in 4 m guanidine HCl. The OD_{280} absorbance was used to determine the apoE-containing fractions, which were then pooled, dialyzed, and lyophilized. Samples were run on 12% SDS gels to confirm the purity of apoE preparation. As a standard for comparison we used murine apoE, kindly provided by Dr. K. H. Weisgraber (San Francisco, CA). Protein concentration of purified apoE was evaluated by a modification to the procedure of Lowry et al. (22).

To determine apoE concentration in serum of C57BL/6 mice, comparative dilution Western blot analyses were performed on a pool of C57BL/6 serum with purified apoE as a standard. Samples were analyzed in triplicate. The concentration of mouse apoE in serum was calculated to be 3.87 mg/dl. The same pool of C57BL/6 serum was used as a control in immunoblots for analysis of apoE concentration in all transplanted mouse serum samples.

Cloning of mouse and human apoE cDNA into the pLXSN retroviral vector

The mouse apoE cDNA (exon 2 through exon 4) was amplified by reverse transcription PCR. The human apoE3 was amplified from an expression vector driven by the CMV promoter (23). Cycling conditions for both the mouse and human apoE consisted of 40 cycles of: 97°C denaturing for 30 sec, 62°C annealing for 2 min, and 72°C extension for 2 min. The PCR products were used for ligation into the pLXSN retroviral vector and transfected into DH5α cells. The sequence of the apoE cDNA inserts was confirmed using the ThermoSequenase kit (Amersham, Arlington Heights, IL).

Preparation of producer cell lines

The retroviral constructs were used to transfect the ecotropic packaging cell line, Bosc-23, which has been shown to result in transient high titer production of infectious virus (24). The supernatant from these cells was used to infect the amphotropic packaging cell line, PA317 (25). The media from this cell line was then used to infect the ecotropic packaging cell line, ψ2, in a trans-infection method for creating high titer producer cell lines (26). The infected ψ2 cells carrying the provirus were selected with the neomycin analogue G418 (Sigma, St. Louis, MO). Individual clones were isolated and analyzed for apoE expression and infectious titer. Mouse apoE-expressing (pLMESN/ψ2), human apoE-expressing (pLHESN/ψ2), and parental virus (pLXSN/ψ2) producer cells were selected for use in bone marrow transplant experiments. The producer cell lines used for BMT studies were assayed for the presence of recombinant wild-type virus by the S^+L^- assay (27, 28) and were found to be helper virus-free.

Bone marrow transplants

Bone marrow was collected from apoE(−/−) and apoE(+/+) mice by flushing femurs and tibias using RPMI 1640 with 2% FBS and 10 U/ml heparin (Sigma). ApoE(+/+) marrow was added to apoE(−/−) marrow at 1, 5, 10, and 25%. Cells were washed, counted, resuspended in RPMI, and used immediately for transplantation into lethally irradiated recipient mice. ApoE(−/−) mice were given 5×10^6 cells in a volume of 300 μl via tail vein injection 4 h after lethal irradiation with 900 rads from a cesium gamma source. Bone marrow transplants with retroviral-transduced marrow were performed as follows: bone marrow was collected from apoE(−/−) donors, counted, and plated in suspension dishes, where proliferation was stimulated by pre-culturing with interleukin 3 (IL-3) and interleukin 6 (IL-6) for 48 h. After pre-culture, bone marrow cells were collected and re-plated at 5×10^5 cells/ml in the above media with 6 μg/ml polybrene (hexadimethrine bromide, Sigma) onto dishes of 80% confluent producer cells again for 48 h. The non-adherent bone marrow cells were then collected and used to transplant lethally irradiated apoE(−/−) mice as described above. Cells were counted, resuspended in RPMI 1640, and injected into the tail vein of recipient mice ($3–5 \times 10^6$ cells in 300 μL).

Serum cholesterol and triglyceride analysis

Blood samples were collected by retro-orbital venous plexus puncture using heparinized tubes. Serum was separated by centrifugation and preserved using 1 mm phenylmethylsulfonyl fluoride (PMSF) (Sigma). Serum cholesterol levels were determined using Raichem reagent No. 80035VI (San Diego, CA), adapted for a microtiter plate assay (29). Briefly, 100 μL of a 1:100 dilution of serum was mixed with 100 μL of reagent and incubated at 37°C for 10 min in a microtiter plate. The 490 nm absorbance was read on a Molecular Devices microplate reader (Menlo Park, CA). Serum triglyceride levels were determined using Sigma Kit No. 339 similarly adapted for microtiter plate assay (absorbance was read at 540 nm).

Lipoprotein separation

Serum samples from individual mice were subjected to fast performance liquid chromatography (FPLC) analysis using a Superose 6 column from Pharmacia on a Waters HPLC system

model 600 (Milford, MA). Briefly, a 100-μL aliquot of serum was injected onto the column and separated with a buffer containing 0.15 m NaCl, 0.01 m Na_2HPO_4, 0.1 mm EDTA, pH 7.5, at a flow rate of 0.5 ml/min. Forty 0.5-ml fractions were collected and tubes 11–40 were analyzed for cholesterol and apoE content. Fractions 14–17 contained VLDL and chylomicrons; fractions 18–25 contained LDL/IDL, fractions 26–31 contained HDL; fractions 32–40 contained the remaining non-lipid bound proteins.

Apolipoprotein E Western blot analyses

Serum samples were tested for the presence of apoE by dot blot analysis. Samples were applied at different dilutions to individual wells of a 96-well dot blot apparatus (Shleicher and Schuell, Keene, NH) along with dilutions of C57BL/6 serum. The primary antibody used was polyclonal rabbit anti-mouse apoE (BioDesign International, Kennebunk, ME). The secondary antibody was horseradish peroxidase-conjugated goat anti-rabbit IgG (Sigma). Signal was detected using the ECL kit by Amersham. Similar Western blot analyses were performed on FPLC samples after electrophoresis on 12% SDS gels and transfer to nylon membranes (Gelman). Serum from pLMESN, pLHESN, and pLXSN were electrophoresed on LFS gels by Z-axis (Hudson, OH), according to the kit instructions.

Densitometric analysis

Densitometric analyses were performed on a MultiImage light cabinet with the AlphaImage 3.24 software (Alpha Innotech Corp., San Leandro, CA). Concentrations of chimeric mouse apoE levels were compared to C57BL/6 concentrations on dot blot analyses. Undiluted apoE-deficient mouse serum was used as a control for cross-reactivity of the polyclonal antibody with other apoproteins. Individual values were then compared to the percent reduction in serum cholesterol from individual mice at 6 weeks

post-BMT. Linear regression was calculated comparing the serum cholesterol reduction with the log of the apoE concentration.

Macrophage primary cultures

Peritoneal cells were collected 2 days after injection of thioglycolate as described (30), counted, and plated into 12-well dishes at 2×10^6 cells/well in DMEM + 10% FBS. After 2 h, non-adherent cells were removed. Wild-type, C57BL/6 mouse peritoneal leukocytes collected and plated in the same manner served as a positive control. Medium was collected from macrophages in 48-h intervals and frozen in 1 mm PMSF for Western blot analysis.

Turnover studies

Lipoproteins of different densities were prepared from serum of triton-treated C57BL/6 or non-treated retroviral-transduced BMT recipient mice. Serum was centrifuged on a Beckman ultra-centrifuge in a TL120 rotor. Density cuts were: chylomicrons (d < 1.006 g/ml), VLDL (d 1.006–1.019 g/ml), and IDL/LDL (d 1.019–1.040 g/ml). Turnover studies were performed essentially as described (31). Groups consisted of 3–4 recipient mice. ^{125}I-labeled lipoproteins were injected via the jugular vein. Blood was collected by retro-orbital venous plexus puncture with heparinized tubes immediately, 15, 60, and 180 min after injection. Aliquots of serum were analyzed for radioactivity on a gamma counter. Total counts were calculated assuming that mouse plasma volume is 3% of body weight.

Negative stains

Plasma lipoproteins were sized by negative stain electron microscopy using carbon-stabilized Formvar-coated nickel grids and 2% aqueous phosphotungstic acid, pH 6.5. Stained grids were viewed in a Philips 300 electron microscope (Philips Electronic Instruments Inc., Mahwah, NJ), and random fields were selected and photographed. Lipoprotein diameters
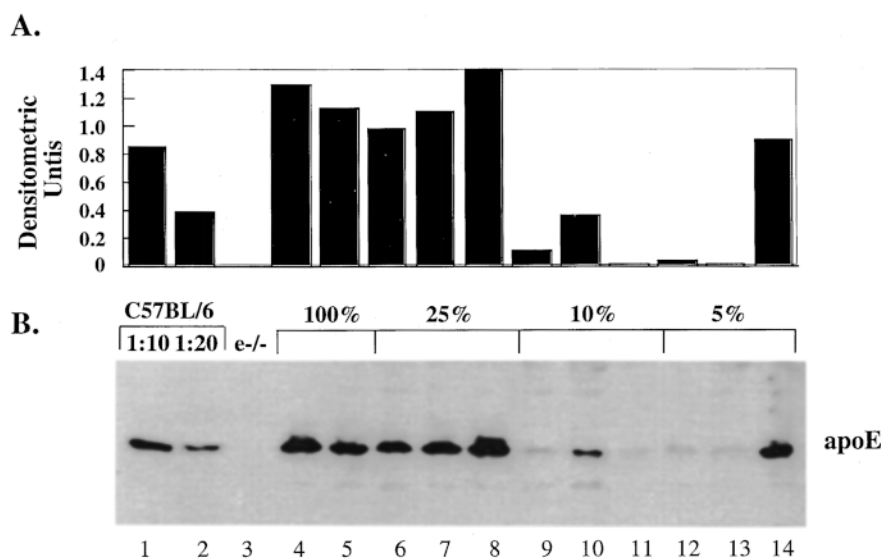

Fig. 1. Western blot analysis of apoE levels in chimeric mice. Serum was collected from recipient mice of apoE(−/−)/apoE(+/+) mixed marrow at 3 weeks after transplantation. SDS PAGE was performed on 3 μl of undiluted serum samples from mice in the different transplantation groups (100, 25, 10, and 5% C57BL/6 marrow) to evaluate the chimerism created by this technique. After electrophoresis on 12% SDS gels, samples were transferred to nitrocellulose membranes and probed for mouse apoE, as described in Materials and Methods. Panel A: densitometric analysis of apoE expression from mouse serum in panel B. Panel B: Lanes 1–2, 1:10 and 1:20 dilutions of C57BL/6 serum; lane 3, apoE(−/−) serum; lanes 4–5, serum from a 100% C57BL/6 recipient; lanes 6–8, serum from 25% recipients; lanes 9–11, serum from 10% recipient mice; lanes 12–14, serum from 5% recipients.
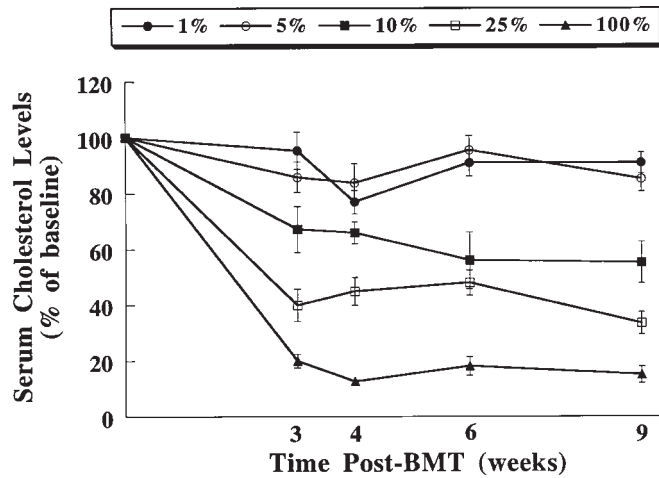
Fig. 2. Serum cholesterol changes in chimeric mice. Serum cholesterol levels were measured in recipient BMT mice before and at 3, 4, 6, and 9 weeks post-BMT. The percent reduction from baseline was determined for individual mice and the results were averaged at each timepoint. The transplantation groups were as follows: 100% C57BL/6 marrow (n = 3), ▲; 25% C57BL/6 marrow (n = 6), □; 10% C57BL/6 marrow (n = 8), ■; 5% C57BL/6 marrow (n = 13), ○; 1% C57BL/6 marrow (n = 4), ●. Error bars represent the standard error of the mean for each group. All timepoints for mice in the 25% and 100% groups were significantly different ($P < 0.001$) from the respective timepoints of mice in both the 1% and 5% groups.

were determined by measuring all spherical lipoproteins within a given field. Between 250 and 500 lipoproteins were measured from three samples in each group (pLHESN and pLXSN). Particles were grouped in two categories: >30 nm and <30 nm. Statistical evaluation of the difference in size distribution between particles of the two groups was performed by Chi-square analysis.

RESULTS

Threshold of serum apoE necessary for serum cholesterol changes

When apoE(−/−) mice are transplanted with 100% wild-type bone marrow, serum apoE concentration is 10–12% of normal levels (0.38–0.46 mg/dl) and serum cholesterol levels are normalized (17, 19, 20). To express lower levels of apoE, we produced chimeric mice by mixing apoE(+/+) and apoE(−/−) bone marrow in different proportions (1, 5, 10, and 25% C57BL/6), and using this marrow to transplant lethally irradiated apoE(−/−) recipient mice. The resulting serum apoE levels in these mice ranged from 0.005 to 0.45 mg/dl. ApoE Western blot analysis of serum 4 weeks after transplantation demonstrated that, although apoE levels tended to be lower with the lower proportion of C57BL/6 marrow in the mixture, there were overlaps of apoE expression between groups (**Fig. 1**). When cholesterol data are compiled for mice based on transplantation groups, mice receiving 10% apoE(+/+) marrow (0.04–0.05 mg/dl) have a 40% reduction in serum cholesterol, whereas mice receiving 5% or 1% mixed marrow (0.005–0.04 mg/dl) do not have significant cholesterol changes (**Fig. 2**). Peritoneal macrophages were collected from mice 6 to 9 months after BMT. Western blot analyses of media from cultured cells demonstrated variability in apoE expression between groups (**Fig. 3**), suggesting that the plasma levels of apoE are a direct reflection of macrophage apoE expression level. To determine the threshold of apoE necessary for cholesterol reductions, individual samples were assayed for apoE concentration and cholesterol reductions. A standard pool of mouse serum with apoE levels measured to be 3.78 mg/dl was used for all comparative dilution immunoblot analyses. There was a significant correlation be-
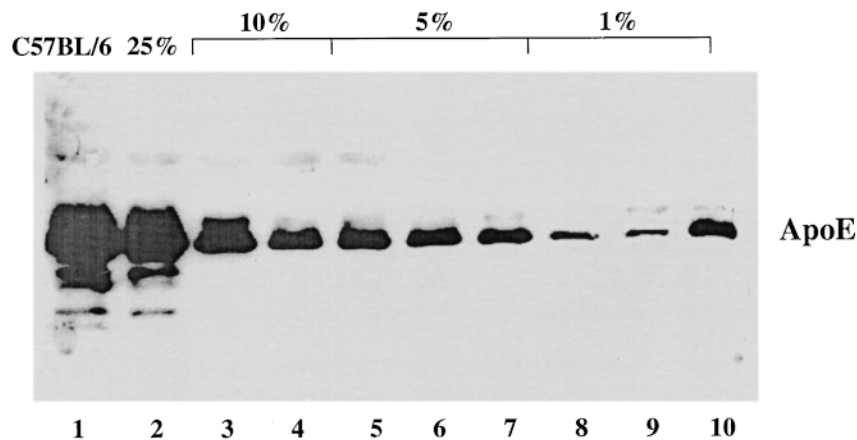

Fig. 3. Western blot analysis of macrophage apoE from chimeric mice. Peritoneal macrophages were collected from BMT recipient mice at killing after thioglycollate injection. Cells were washed and plated onto 12-well dishes at 1×10^6 cells per well. After macrophages had seeded, they were washed and the media were collected in 48-h intervals. Lipoproteins were extracted from the media with fumed silica and electrophoresed on 12% SDS gels. Proteins were transferred to nitrocellulose membranes and probed for apoE. The protein content of individual wells was quantitated by a Lowry assay and equivalent amounts of media per mg cell protein were loaded onto the gel. Lane 1, C57BL/6 macrophages; lane 2, 25% mouse; lanes 3–4, 10% mice; lanes 5–7, 5% mice and lanes 8–10, 1% mice.
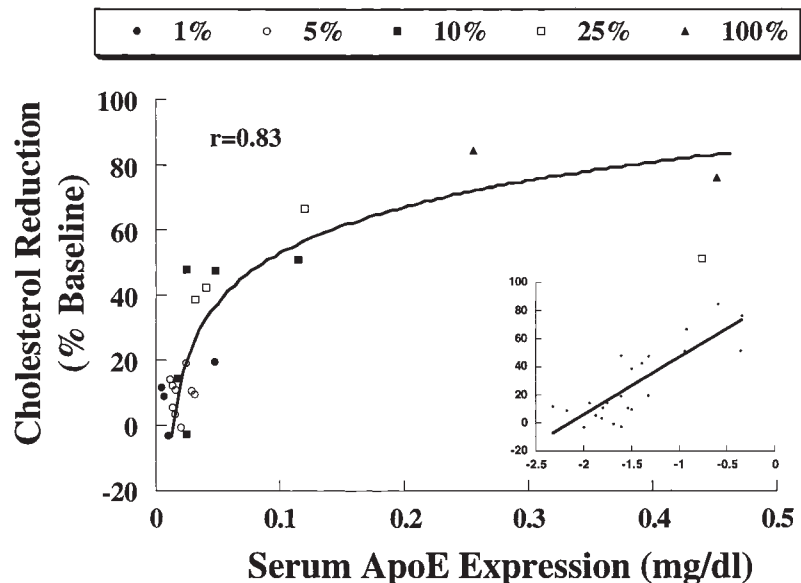
Fig. 4. Correlation between serum apoE and cholesterol reduction in chimeric mice 6 weeks after BMT. Serum cholesterol levels at baseline and 6 weeks after transplantation were evaluated as described in Materials and Methods. Serum samples were then used for dot blot and densitometric analysis and compared with serial dilutions of C57BL/6 serum (described in Methods). Samples were analyzed in triplicate and individual values for serum apoE levels were compared to serum cholesterol reductions. A logarithmic correlation line was plotted through the data. The inset contains a graph demonstrating the linear correlation ($r = 0.83$) between the cholesterol reduction and the log of the apoE concentration for individual samples. The transplantation groups of individual mice are indicated by symbols: 100% C57BL/6 marrow (n = 2), ▲; 25% C57BL/6 marrow (n = 4), □; 10% C57BL/6 marrow (n = 5), ■; 5% C57BL/6 marrow (n = 9), ○; 1% C57BL/6 marrow (n = 3), ●.

tween serum cholesterol reductions and the log of the apoE levels ($r = 0.83$). The threshold of apoE below which no changes in cholesterol were observed was about 0.04 mg/dl (**Fig. 4**).

Remnant lipoproteins are progressively reduced as apoE levels increase

Lipoprotein profiles of chimeric mice with different serum levels of apoE were evaluated by FPLC analysis. Analyses were performed on 5–7 individual samples from each group. The changes in lipoprotein profiles were characterized first by reductions in the VLDL and IDL fractions, followed by reductions in LDL (**Fig. 5**, panel A). The distribution of apoE on lipoproteins was determined by Western blot analysis of FPLC fractions. The apoE was located primarily on VLDL, IDL, and LDL, with smaller amounts on HDL (Fig. 5, panel B) in all groups of mice.

Clearance of IDL in the presence of low level apoE

Due to the variability in apoE concentrations produced in the chimeric BMT mice, we used another technique to obtain mice with consistent low levels of apoE in serum. ApoE(−/−) mice were transplanted with apoE(−/−) marrow that had been transduced with either a mouse or human apoE-containing retrovirus (pLMESN and pLHESN, respectively). These mice had levels of 0.02 mg/dl apoE in serum, which was consistent between mice and was always below the threshold necessary to induce measurable cholesterol changes (**Fig. 6**). Serum apoE concentrations and cholesterol levels in mice transplanted with transduced marrow are stable for up to 16 weeks after transplantation (not shown). Controls used for these experiments were mice transplanted with parental-virus transduced cells (pLXSN).

To determine whether serum apoE concentrations below the cholesterol reduction threshold would affect the clearance of remnant lipoproteins, we studied the turnover of ^{125}I-labeled VLDL (d < 1.019 g/ml) from C57BL/6

or pLMESN mouse plasma in three groups of mice: C57BL/6, pLXSN, and pLMESN. Serum samples were collected at 15, 60, and 180 min after injection. The clearance of both VLDL types was greater than 80% in C57BL/6 mice by 3 h after injection, but was equally delayed in
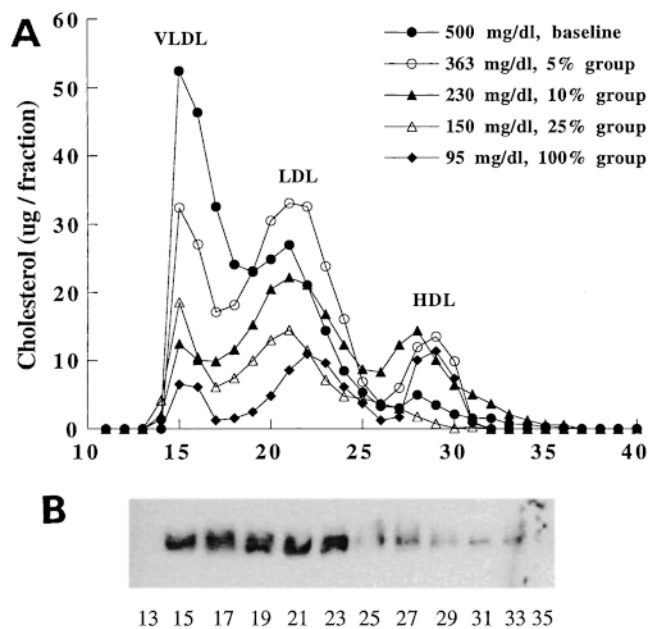

Fig. 5. FPLC analysis of lipoprotein distribution in serum of chimeric mice. Individual mouse samples with increasingly reduced total cholesterol values were analyzed by FPLC analysis. Samples were injected onto a Superose-6 column and separated into forty 0.5-ml fractions as described in Materials and Methods. Cholesterol levels were evaluated for each sample and plotted. Panel A: Lipoprotein distribution of mice from baseline (●, 500 mg/dl), 5% recipient (○, 363 mg/dl), 10% recipient (▲, 230 mg/dl), 25% recipient (△, 150 mg/dl), and 100% recipient (◆, 95 mg/dl). Representative examples of one animal from each group are presented. Panel B: Western blot analysis of apoE in FPLC fractions from a 5% recipient mouse.
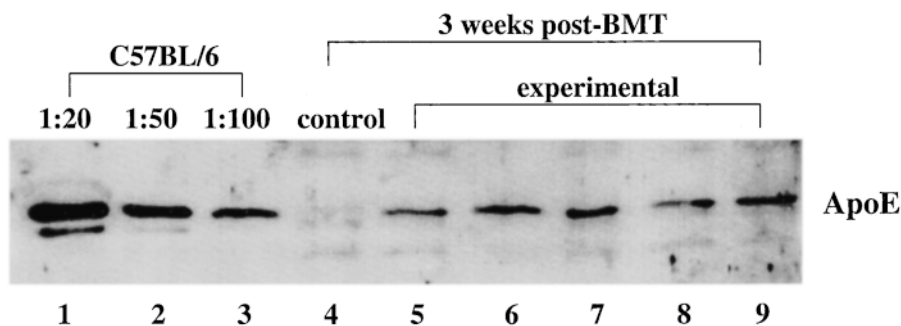
Fig. 6. ApoE Western blot analysis in serum of apoE(−/−) mice transplanted with apoE(−/−) bone marrow transduced with an apoE-expressing retrovirus. Three-μL aliquots of fasted serum samples were subjected to 12% SDS-PAGE. Proteins were transferred to a pure nitrocellulose membrane (Gelman) by semidry transfer and immunoblotted as described in Materials and Methods. Lanes 1–3 contain 3 μL of C57BL/6 serum as stated, lane 4 is serum from a control mouse transplanted with apoE(−/−) bone marrow transduced with an empty retrovirus, and lanes 5–9 contain serum from 5 different experimental mice transplanted with pLMESN.

pLMESN and pLXSN mice, with 40% of the initial counts still in serum 3 h after injection (data not shown).

In addition, the clearance of different remnant lipoprotein classes was tested by injecting C57BL/6 ^{125}I-labeled chylomicrons (d < 1.006 g/ml), VLDL (d 1.006–1.019 g/ml), and LDL (d 1.019–1.040 g/ml) into pLXSN and pLHESN mice. Again, no differences were detected between mice expressing low levels of extrahepatic apoE compared to control pLXSN mice (**Fig. 7**). Interestingly, there was no difference in the clearance of LDL between apoE(−/−) and C57BL/6 mice, presumably because this particle binds to the LDLR via apoB-100 (Fig. 7, panel C). Non-denaturing gel electrophoresis was also performed on serum from pLHESN, pLMESN, and pLXSN to determine whether any differences in remnant concentrations were detectable. A broad VLDL band was evident in all samples but no differences were distinguishable among groups (data not shown).

Finally, the size distribution of serum lipoproteins from fasted pLXSN and pLHESN mice was analyzed by electron microscopy after negative staining. Lipoprotein particles from each group were separated into those >30 nm (VLDL and chylomicron remnants) and those <30 nm (mostly IDL and LDL). Mice expressing low levels of apoE had a significant reduction in lipoproteins with diameters less than 30 nm ($P = 0.011$ by Chi-square analysis, **Fig. 8**), indicating a possible preferential loss of these smaller remnant particles (<30 nm) when apoE is present in limiting concentrations. Similar results were obtained with pLMESN mouse serum.

DISCUSSION

The goals of this study were to find the threshold level of apoE necessary to produce measurable effects on serum
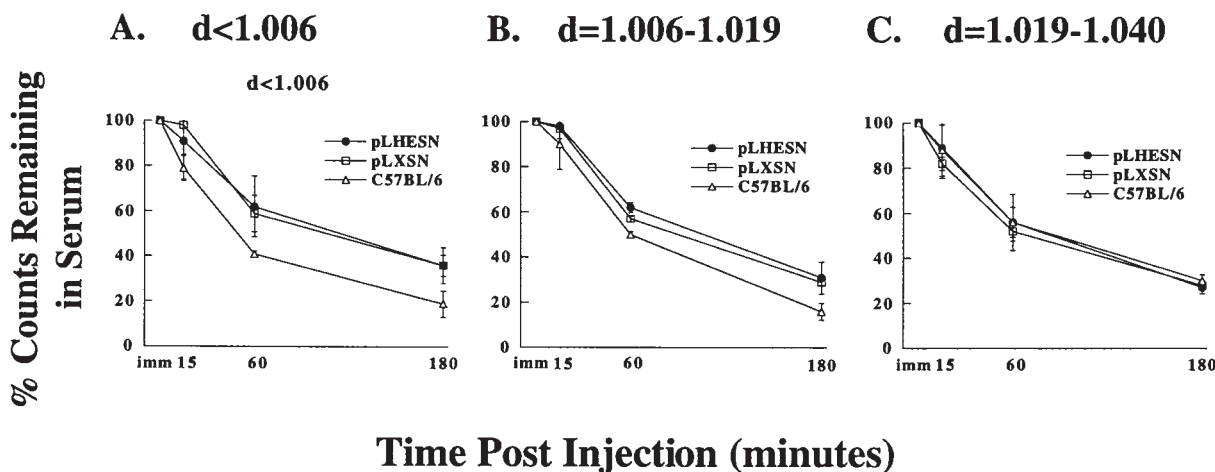

Fig. 7. Lipoprotein turnover studies in C57BL/6, pLHESN and pLXSN mice. Chylomicrons (d < 1.006 g/ml), VLDL (d 1.006–1.019 g/ml), and LDL (d 1.019–1.040 g/ml) were labeled with ^{125}I and used for turnover studies in C57BL/6, pLHESN, and pLXSN mice (n = 3 in each group). Injections were made in the jugular bulb and blood collections were performed through the orbital venous plexus immediately after injection and at 15, 60, and 120 min after injection. Counts in serum are calculated as the percent of the immediate counts. Panel A demonstrates the turnover of chylomicron remnants, panel B is of VLDL, and panel C is of LDL.
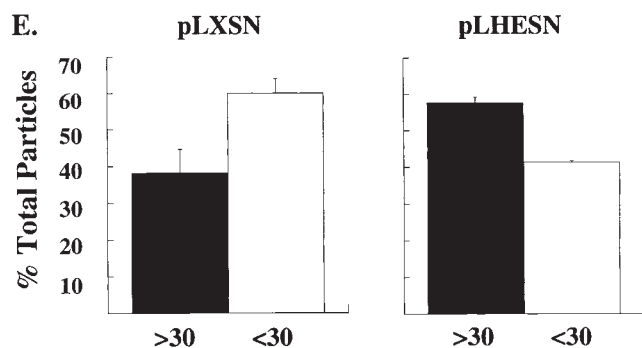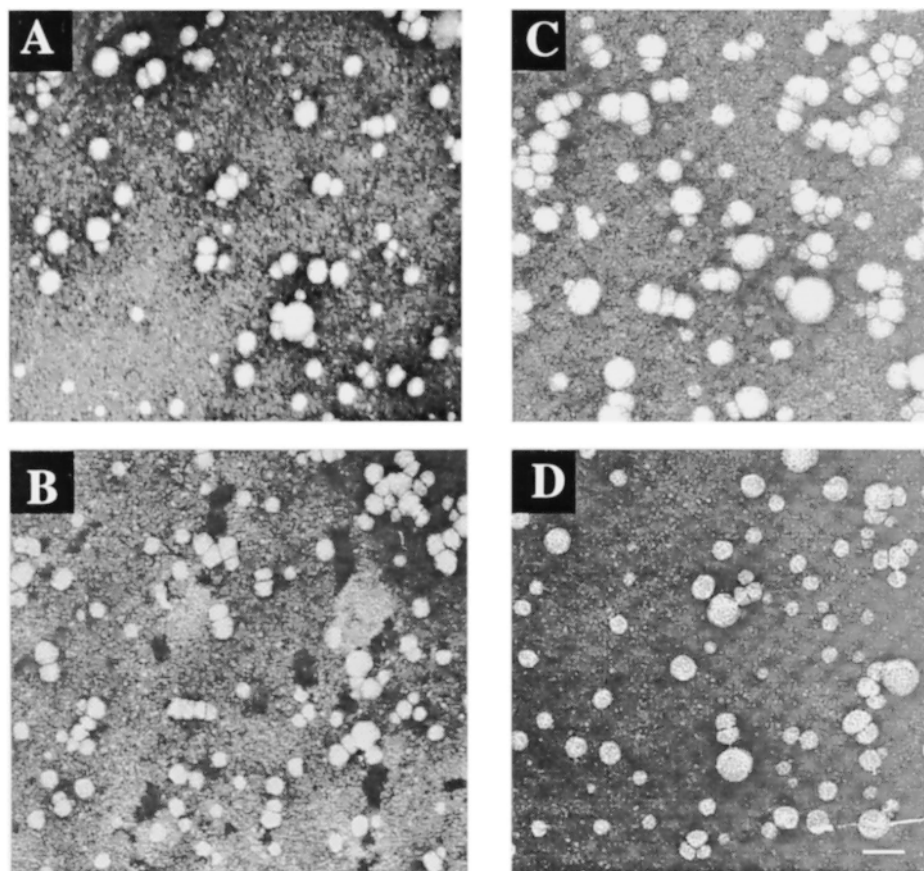
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Fig. 8. Negative stain analysis of serum lipoproteins in pLXSN and pLHESN mice. Blood samples were collected and serum was centrifuged from mice that had been fasted for 12 h to control for the presence of post-prandial lipoproteins. Negative stains were prepared and visualized by electron microscopy according to Materials and Methods. The diameters of the particles were converted to nm based upon the magnification of the picture taken. Panels A and B are representative images from two pLXSN mice, and panels C and D are from two pLHESN mice. Bar indicates 100 nm. Panel E presents the relative distribution of particle sizes above and below 30 nm (n = 3 for each group). There was a significant difference in the distribution of lipoproteins from the two groups of mice ($P = 0.011$, 2×2 Chi-square analysis).

cholesterol concentrations and to investigate whether any subtle effects on remnant lipoprotein clearance could be detected below this level. To evaluate the lower threshold of apoE for lipoprotein clearance, mice with different concentrations of apoE were produced by transplanting apoE(−/−) mice with different proportions of marrow from C57BL/6 and apoE(−/−) mice. The resulting chimeric mice expressed apoE at various levels allowing us to analyze the threshold of apoE in serum which would still result in cholesterol clearance. The relationship between the log of serum apoE levels and cholesterol reduction was highly significant ($r = 0.83$). Serum apoE levels below 0.04 mg/dl resulted in small, variable, and non-significant changes in serum cholesterol levels whereas apoE concentrations between 0.04 and 0.08 mg/dl resulted in cholesterol reductions ranging from 20 to 50%. At apoE concen-

trations greater than 0.1 mg/dl, cholesterol levels were nearly normalized, with cholesterol reductions in the order of 60–80%. Thus, the threshold of apoE necessary for detectable cholesterol clearance is 0.04 mg/dl. This is approximately 100-fold lower than normal levels of apoE in serum. In addition, our negative stain analyses of serum from mice with apoE levels below the threshold indicates that a population of smaller remnant particles (diameter <30 nm) is selectively under-represented. As apoE is an exchangeable apoprotein that transfers from one lipoprotein particle to another, it is possible that the exchange of apoE among different lipoproteins allows IDL-size particles, which have a smaller surface-to-core ratio than other remnants, to accumulate the critical mass of apoE necessary for their clearance more readily than the larger VLDL and chylomicrons (a schematic representation of
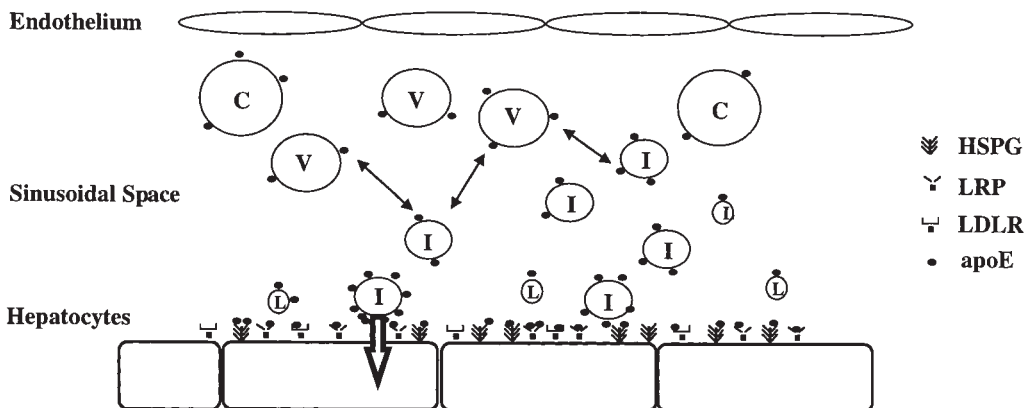
Fig. 9. Diagram of the hypothesized effects of low levels of apoE in serum. In this schematic representation, limiting levels of apoE in serum are responsible for reduced apoE content on lipoproteins in the space of Disse. We hypothesize that the exchange of apoE between lipoprotein particles in the space of Disse allows smaller IDL-size remnants to accrue the critical mass of apoE necessary for their clearance by hepatic receptors. The apoE originating from apoE-producing Kupffer cells may have a particularly relevant role in this pathway. The arrow indicates internalization of an apoE-enriched IDL. (C, chylomicrons; V, VLDL; I, IDL; L, LDL).

this hypothesis is given in **Fig. 9**). In this scenario, it is intriguing to speculate that the apoE-producing Kupffer cells of donor origin may preferentially enrich smaller remnants and accelerate their hepatic clearance. Another interpretation is that the change in lipoprotein particle distribution reflects increased levels of larger remnants due to reduced processing of chylomicrons secondary to apoE inhibition of lipoprotein lipase (32, 33). However, the lack of changes in triglyceride levels between apoE-expressing and apoE(−/−) mice argues against this interpretation.

The data presented here lead to a clearer understanding of the relationship between serum apoE and cholesterol concentrations. Much work has been done to evaluate the effects of over-expression of apoE in mice. Both adenoviral delivery and transgenic approaches have been used to evaluate the effects of over-expression of apoE in mice. Adenoviral delivery of human apoE in apoE(−/−) mice has been reported to result in serum expression of levels as high as 650 mg/dl, although levels of only 1–2 mg/dl were needed to reduce serum cholesterol levels (34, 35). When apoE is expressed at very high levels, serum cholesterol levels are actually increased, presumably due to the competition of free apoE with lipoprotein-bound apoE for hepatic receptors. Shimano et al. (36) have produced mice expressing rat apoE under the control of a metallothionein promoter at levels greater than 10 mg/dl. This high level of apoE expression not only reduced serum cholesterol and triglyceride levels, but it also reduced the progression of atherosclerosis in animals on a high fat diet. Together with our data, a scenario can be depicted where a broad range of apoE concentrations (from 0.1 to 10 mg/dl) is compatible with the maintenance of normal serum cholesterol homeostasis. Our data demonstrate that at lower concentrations of apoE (from 0.04 to 0.1 mg/dl), there is a dose-dependent relationship between apoE and serum cholesterol levels. Below the level of 0.04 mg/dl, apoE is no longer capable of promoting

bulk lipoprotein clearance and cholesterol reduction, but is associated with a different lipoprotein size distribution compared to the apoE-deficient state.

The reported concentrations of serum apoE after BMT with wild-type marrow have varied from undetectable (20), to 3.8% of normal (19), to our report of 10–12% of normal (17). We and another group have also reported the effects of transplantation of apoE(−/−) mice with apoE(+/−) marrow. Although we both reported a significant 50% reduction in serum cholesterol levels following BMT, we estimated apoE levels to be 5% of normal (17), whereas VanEck at al. (19) calculated a concentration of 1.87% of normal. Therefore, the discrepancy between the two reports most likely reflects methodological differences.

It is interesting to note that human and mouse apoE appear to have different efficacy in inducing lipoprotein clearance in mice. When Bellosta et al. (37) crossed apoE(−/−) mice with transgenic mice expressing human apoE under the control of a macrophage specific promoter, the resulting animals had plasma apoE levels ranging from 0.02 to 1.1 mg/dl. Levels below 0.4 mg/dl were not sufficient to induce serum cholesterol changes, whereas levels above 0.4 mg/dl reduced plasma cholesterol levels by 30–60%. In contrast, we report here that mouse apoE need only be present in serum levels of 0.04 mg/dl (10-fold less than human apoE3) to result in serum cholesterol reductions. Other reports confirm the difference in efficacy between mouse and human apoE in the mouse system. Tsukamoto et al. (38) used second generation adenovirus expressing human apoE in apoE(−/−) mice and found that 30% of normal concentrations of human apoE were required for serum cholesterol normalization. Sullivan et al. (39, 40) produced mice in which the endogenous apoE gene was homologously replaced with the human apoE2 or apoE3 genes. In these mice, human apoE levels were identical to that of wild-type mouse apoE levels; however, both human apoE-expressing

mice had delayed clearance of apoE($-/-$) VLDL and a dramatic increase in serum cholesterol levels in response to a high fat, high cholesterol diet when compared to wild type mice. Moreover, atherosclerotic lesion development was negatively affected by the expression of apoE3 and, particularly, apoE2 in these mice. These data provide evidence for the differing effects of mouse and human apoE in the mouse model.

An interesting question raised by these studies is why there is such an apparent excess of apoE in serum. One possibility is related to the apoE secretion–capture hypothesis (42–44). Secretion–capture is a mechanism by which apoE secreted by hepatocytes is sequestered in the space of Disse, attached to the HSPG. This hepatocyte-bound apoE is thought to assist in the capture of incoming remnant lipoproteins, and to promote their removal from the circulation. In this model, excess apoE would be beneficial in circumstances such as in the presence of postprandial dietary fatty acid intake or when the LDL receptor is not functioning properly. A recent study in our laboratory implicates the LRP as playing a role in lipoprotein uptake only in the presence of hepatic secretion of apoE (41). The physiologic over-expression of apoE may serve to route lipoprotein uptake through the LRP to ensure appropriate intracellular handling.

In conclusion, we present evidence for significant biologic redundancy of apoE in serum. ApoE levels ranging from the physiologic concentration of ~4 mg/dl to the low level of 0.4 mg/dl are compatible with complete plasma cholesterol homeostasis. Below this level, there is a range of concentrations in which apoE has a dose-dependent effect on serum cholesterol levels. At levels lower than 0.04 mg/dl, apoE is no longer capable of inducing detectable serum cholesterol changes, but subtle effects on remnant clearance continue to occur. These results indicate that even extremely low levels of serum apoE have a functional role in lipoprotein removal, and are suggestive of a hierarchical clearance of different lipoproteins when serum apoE is in limiting amounts.

REFERENCES

1. Mahley, R. W. 1988. Apolipoprotein E: cholesterol transport protein with expanding role in cell biology. *Science.* **240:** 622–630.
2. Mahley, R. W., K. H. Weisgraber, M. M. Hussain, B. Greenman, M. Fisher, T. Vogel, and M. Gorecki. 1989. Intravenous infusion of apolipoprotein E accelerates clearance of plasma lipoproteins in rabbits. *J. Clin. Invest.* **83:** 2125–2130.
3. Yamada, N., D. M. Shames, J. B. Stoudemire, and R. J. Havel. 1986. Metabolism of lipoproteins containing apolipoprotein B-100 in blood plasma of rabbits: heterogeneity related to the presence of apolipoprotein E. *Proc. Natl. Acad. Sci. USA.* **83:** 3479–3483.
4. Mahley, R. W., K. H. Weisgraber, and T. L. Innerarity. 1979. Interaction of plasma lipoproteins containing apolipoproteins B and E with heparin and cell surface receptors. *Biochim. Biophys. Acta.* **575:** 81–91.
5. Weisgraber, K. H., S. C. Rall, Jr., R. W. Mahley, R. W. Milne, Y. L. Marcel, and J. T. Sparrow. 1986. Human apolipoprotein E. Determination of the heparin binding sites of apolipoprotein E3. *J. Biol. Chem.* **261:** 2068–2076.
6. Rall, S. C., Jr., and R. W. Mahley. 1992. The role of apolipoprotein E genetic variants in lipoprotein disorders. *J. Intern. Med.* **231:** 653–659.
7. Havel, R. J., and J. P. Kane. 1973. Primary dysbetalipoproteinemia: predominance of a specific apoprotein species in triglyceride-rich lipoproteins. *Proc. Natl. Acad. Sci. USA.* **70:** 2015–2019.
8. Ghiselli, G., E. J. Schaefer, P. Gascon, and H. B. Brewer, Jr. 1981. Type III hyperlipoproteinemia associated with apolipoprotein E deficiency. *Science.* **214:** 1239–1241.
9. Mahley, R. W., and S. C. Rall, Jr. 1989. Type III hyperlipoproteinemia (dysbetalipoproteinemia): the role of apolipoprotein E in normal and abnormal lipoprotein metabolism. *In* The Metabolic Basis of Inherited Disease. 6th edition. Vol. I. C. R. Scriver, A. L. Beaudet, W. S. Sly, and D. Valle, editors. McGraw-Hill, Inc., New York. 1195–1213.
10. Piedrahita, J. A., S. H. Zhang, J. R. Hagaman, P. M. Oliver, and N. Maeda. 1992. Generation of mice carrying a mutant apolipoprotein E gene inactivated by gene targeting in embryonic stem cells. *Proc. Natl. Acad. Sci. USA.* **89:** 4471–4475.
11. Plump, A. S., J. D. Smith, T. Hayek, K. Aalto-Setälä, A. Walsh, J. G. Verstuyft, E. M. Rubin, and J. L. Breslow. 1992. Severe hypercholesterolemia and atherosclerosis in apolipoprotein E-deficient mice created by homologous recombination in ES cells. *Cell.* **71:** 343–353.
12. Zhang, S. H., R. L. Reddick, J. A. Piedrahita, and N. Maeda. 1992. Spontaneous hypercholesterolemia and arterial lesions in mice lacking apolipoprotein E. *Science.* **258:** 468–471.
13. Nakashima, Y., A. S. Plump, E. W. Raines, J. L. Breslow, and R. Ross. 1994. ApoE-deficient mice develop lesions in all phases of atherosclerosis throughout the arterial tree. *Arterioscler. Thromb.* **14:** 133–140.
14. Lusis, A. J., B. A. Taylor, D. Quon, S. Zollman, and R. C. LeBoeuf. 1987. Genetic factors controlling structure and expression of apolipoproteins B and E in mice. *J. Biol. Chem.* **262:** 7594–7604.
15. Mahley, R. W., and T. L. Innerarity. 1983. Lipoprotein receptors and cholesterol homeostasis. *Biochim. Biophys. Acta.* **737:** 197–222.
16. Schaefer, E. J., R. E. Gregg, G. Ghiselli, T. M. Forte, J. M. Ordovas, L. A. Zech, and H. B. Brewer, Jr. 1986. Familial apolipoprotein E deficiency. *J. Clin. Invest.* **78:** 1206–1219.
17. Linton, M. F., J. B. Atkinson, and S. Fazio. 1995. Prevention of atherosclerosis in apoE deficient mice by bone marrow transplantation. *Science.* **267:** 1034–1037.
18. Fazio, S., and M. F. Linton. 1996. Murine bone marrow transplantation as a novel approach to studying the role of macrophages in lipoprotein metabolism and atherogenesis. *Trends Cardiovasc. Med.* **6:** 58–65.
19. VanEck, M., N. Herijgers, J. Yates, N. J. Pearce, P. M. Hoogerbrugge, P. H. E. Groot, and T. J. C. VanBerkel. 1997. Bone marrow transplantation in apolipoprotein E-deficient mice: effect of apoE gene dosage on serum lipid concentrations, (beta)VLDL catabolism, and atherosclerosis. *Arterioscler. Thromb. Vasc. Biol.* **17:** 3117–3126.
20. Boisvert, W. A., J. Spangenberg, and L. K. Curtiss. 1995. Treatment of severe hypercholesterolemia in apolipoprotein E-deficient mice by bone marrow transplantation. *J. Clin. Invest.* **96:** 1118–1124.
21. Rall, S. C., Jr., K. H. Weisgraber, and R. W. Mahley. 1986. Isolation and characterization of apolipoprotein E. *Methods Enzymol.* **128:** 273–287.
22. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193:** 265–275.
23. Fazio, S., Y. Horie, K. H. Weisgraber, L. M. Havekes, and S. C. Rall, Jr. 1993. Preferential association of apolipoprotein E Leiden with very low density lipoproteins of human plasma. *J. Lipid Res.* **34:** 447–453.
24. Pear, W. S., G. P. Nolan, M. L. Scott, and D. Baltimore. 1993. Pro-

duction of high-titer helper-free retroviruses by transient transfection. *Proc. Natl. Acad. Sci. USA.* **90:** 8392–8396.

25. Miller, A. D., and C. Buttimore. 1986. Redesign of retrovirus packaging cell lines to avoid recombination leading to helper virus production. *Mol. Cell Biol.* **6:** 2895–2902.

26. Mann, R., R. C. Mulligan, and D. Baltimore. 1983. Construction of a retrovirus packaging mutant and its use to produce helper-free defective retrovirus. *Cell.* **33:** 153–159.

27. Brandt, S. J., D. M. Bodine, C. E. Dunbar, and A. W. Nienhuis. 1990. Dysregulated interleukin 6 expression produces a syndrome resembling Castleman's Disease in mice. *J. Clin. Invest.* **86:** 592–599.

28. Bassin, R. H., N. Tuttle, and P. J. Fishinger. 1971. Rapid cell culture assay technique for murine leukaemia viruses. *Nature.* **229:** 564–566.

29. Fazio, S., Y-L. Lee, Z-S. Ji, and S. C. Rall, Jr. 1993. Type III hyperlipoproteinemic phenotype in transgenic mice expressing dysfunctional apolipoprotein E. *J. Clin. Invest.* **92:** 1497–1503.

30. Cohn, Z. A., and B. Benson. 1965. The differentiation of mononuclear phagocytes. Morphology, cytochemistry, and biochemistry. *J. Exp. Med.* **121:** 153–170.

31. Fazio, S., V. R. Babaev, A. B. Murray, A. H. Hasty, K. J. Carter, L. A. Gleaves, J. B. Atkinson, and M. F. Linton. 1997. Increased atherosclerosis in mice reconstituted with apolipoprotein E null macrophages. *Proc. Natl. Acad. Sci. USA.* **94:** 4647–4652.

32. Rensen, P. C. N., and T. J. C. vanBerkel. 1996. Apolipoprotein E effectively inhibits lipoprotein lipase-mediated lipolysis of chylomicron-like triglyceride-rich lipid emulsions in vitro and in vivo. *J. Biol. Chem.* **271:** 14791–14799.

33. Jong, M. C., V. E. H. Dahlmans, M. H. Hofker, and L. M. Havekes. 1997. Nascent very-low-density lipoprotein triacylglycerol hydrolysis by lipoprotein lipase is inhibited by apolipoprotein E in a dose-dependent manner. *Biochem. J.* **328:** 754–750.

34. Stevenson, S. C., J. Marshall-Neff, B. Teng, C. B. Lee, S. Roy, and A. McClelland. 1995. Phenotypic correction of hypercholesterolemia in apoE-deficient mice by adenovirus-mediated in vivo gene transfer. *Arterioscler. Thromb.* **15:** 479–484.

35. Kashyap, V. S., S. Santamarina-Fojo, D. R. Brown, C. L. Parrott, D. Applebaum-Bowden, S. Meyn, G. Talley, B. Paigen, N. Maeda, and H. B. Brewer. 1995. Apolipoprotein E deficiency in mice: gene replacement and prevention of atherosclerosis using adenovirus vectors. *J. Clin. Invest.* **96:** 1612–1620.

36. Shimano, H., N. Yamada, M. Katsuki, M. Shimada, T. Gotoda, K. Harada, T. Murase, C. Fukazawa, F. Takaku, and Y. Yazaki. 1992. Overexpression of apolipoprotein E in transgenic mice: marked reduction in plasma lipoproteins except high density lipoprotein and resistance against diet-induced hypercholesterolemia. *Proc. Natl. Acad. Sci. USA.* **89:** 1750–1754.

37. Bellosta, S., R. Mahley, D. Sanan, J. Murata, D. Newland, J. Taylor, and R. Pitas. 1995. Macrophage-specific expression of human apolipoprotein E reduces atherosclerosis in hypercholesterolemic E-null mice. *J. Clin. Invest.* **96:** 2170–2179.

38. Tsukamoto, K., P. Smith, J. M. Glick, and D. J. Rader. 1997. Liver-directed gene transfer and prolonged expression of three major human apoE isoforms in apoE-deficient mice. *J. Clin. Invest.* **100:** 107–114.

39. Sullivan, P. M., H. Mezdour, S. H. Quarfordt, and N. Maeda. 1998. Type III hyperlipoproteinemia and spontaneous atherosclerosis in mice resulting from gene replacement of mouse apoE with human apoE2. *J. Clin. Invest.* **102:** 130–135.

40. Sullivan, P. M., H. Mezdour, Y. Arantani, C. Knouff, J. Najibi, R. L. Reddick, S. H. Quarfordt, and N. Maeda. 1997. Targeted replacement of the mouse apolipoprotein E gene with the common human apoE3 allele enhances diet-induced hypercholesterolemia and atherosclerosis. *J. Biol. Chem.* **272:** 17972–17980.

41. Linton, M. F., A. H. Hasty, V. R. Babaev, and S. Fazio. 1998. Hepatic apoE expression is required for remnant lipoprotein clearance in the absence of the low density lipoprotein receptor. *J. Clin. Invest.* **101:** 1726–1736.

42. Ji, Z-S., S. Fazio, Y-L. Lee, and R. W. Mahley. 1994. Secretion-capture role for apolipoprotein E in remnant lipoprotein metabolism involving cell surface heparan sulfate proteoglycans. *J. Biol. Chem.* **269:** 2764–2772.

43. Shimano, H., Y. Namba, J. Ohsuga, M. Kawamura, K. Yamamoto, M. Shimada, T. Gotoda, K. Harada, Y. Yazaki, and N. Yamada. 1994. Secretion-recapture process of apolipoprotein E in hepatic uptake of chylomicron remnants in transgenic mice. *J. Clin. Invest.* **93:** 2215–2223.