Role of Apolipoprotein E on Cholesteryl Ester–Enriched Low Density Lipoprotein Particles in Coronary Artery Atherosclerosis of Hypercholesterolemic Nonhuman Primates

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Significant differences among individuals occur in the lipoprotein response to atherogenic diets in cynomolgus and African green monkeys. The range of concentrations of total plasma cholesterol (TPC) was 100–600 mg/dl and of apolipoprotein (apo) E (quantified by enzyme-linked immunosorbent assay) was 3–20 mg/dl in the animal groups of this study. The correlation between the concentrations of TPC and of apo E was r=0.89 in these animals. To determine which lipoprotein classes contained the majority of apo E, agarose gel-filtration chromatography was used to subfractionate whole plasma. In hypercholesterolemic monkeys, the majority of the apo E and apo B-100 coeluted within the region of low density lipoprotein (LDL). In normcholesterolemic monkeys, the majority of apo E coeluted with apo A-I and high density lipoproteins. A strong positive correlation was seen between the concentrations of plasma apo E and LDL cholesterol (r=0.9), but there was no significant correlation between high density lipoprotein apo E and either TPC or plasma apo E concentrations. A positive correlation of r=0.8 was found between the LDL apo E to apo B-100 molar ratio and the average LDL particle size, suggesting an increase in the number of apo E molecules on the larger LDL particles. Within individual animals, LDL were heterogeneous and the LDL subfractions of lower density (1.02<d<1.03 g/ml) had the highest proportion of apo E, although apo E was present on LDL of all densities. A strong positive correlation between plasma apo E concentration and coronary artery atherosclerosis was identified, and in stepwise regression analysis, apo E concentration and the apo E to apo B molar ratio of LDL together accounted for more than 90% of the variation in the atherosclerosis end point of coronary artery intimal area. These data strongly suggest that the enrichment of LDL with cholesteryl esters and apo E, which occurs in hypercholesterolemic primates, is an atherogenic feature of the plasma lipoproteins. (Arteriosclerosis and Thrombosis 1992;12:28–40)

Apolipoprotein E (apo E) is present on almost all lipoproteins and is thought to play a role in cholesterol transport by mediating the receptor-dependent clearance of various lipoproteins, including intestinally derived chylomicrons as well as very low density lipoproteins (VLDL), intermediate density lipoproteins (IDL), low density lipoproteins (LDL), and high density lipoproteins (HDL).1−3 Chylomicron remnant clearance is dependent on the interaction of apo E with a hepatic apo E-specific receptor that is now thought to be the LDL receptor–related protein, or LRP.4,5 Apo E has a higher affinity for the LDL receptor than does apo B-100,6 and the former has been shown to aid in the receptor mediated-clearance of VLDL, IDL, and LDL. Studies of several animal models, including cynomolgus monkeys,7 New Zealand White rabbits,8 and Watanabe heritable hyperlipidemic (WHHL) rabbits,9,10 demonstrate that VLDL, IDL, and LDL have an increased rate of clearance from plasma when apo E is present on the lipoprotein particle. In species without cholesterol ester transfer protein...
activity such as the dog and the rat, apo E is thought to be involved in reverse cholesterol transport through the formation and subsequent hepatic removal of cholesteryl ester–rich apo E–HDL. 15,16,21 In cases of apo E deficiency, it has been shown that there is an accumulation and abnormal clearance of chylomicron remnants, VLDL, and LDL. 12-14 From these studies, apo E appears to play an important role in the metabolism of most lipoproteins.

The distribution of apo E among the plasma lipoproteins and the factors affecting this distribution in humans and several animal models have been studied. 15-24 Hypercholesterolemia induced by dietary cholesterol is one factor that can influence the apo E distribution among the plasma lipoproteins. Early studies of several animal models, including the patas monkey, 15 rat, 16 rabbit, 17 and pig, 18 documented a qualitative change in the apo E distribution among lipoprotein classes after feeding diets enriched in cholesterol. With the induction of hypercholesterolemia, there was an enrichment of apo E in VLDL and HDL as determined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Studies employing more quantitative measures, such as electroimmunoassay for rhesus monkeys 22 and the radioimmunoassay for humans 19-20 and rats, 11,21 have also reported an increased proportion of apo E associated with VLDL and HDL due to the induction of hypercholesterolemia. However, in most of these studies, the use of repeated ultracentrifugation steps to isolate lipoproteins may have modified the outcome, as apo E disassociates from lipoproteins under these conditions. 10,18-21

We have consistently observed large differences among individual monkeys in their lipoprotein response to an atherogenic diet. 25-28 In a group of 40 cynomolgus monkeys fed the same amount of cholesterol, total plasma cholesterol (TPC) concentrations varied from 150 to 800 mg/dl. 27 In African green as well as cynomolgus monkeys, the lipoprotein cholesterol distribution is proportional to the TPC concentration, with higher HDL and lower LDL cholesterol concentrations at lower TPC. As TPC concentrations increase, progressive decreases in HDL and increases in LDL cholesterol concentrations are found. 28 We have seen a difference in apo E content among individual LDL subpopulations. 30 To evaluate the shift in the apo E distribution that occurs with the shift in lipoprotein distribution, the apolipoprotein distributions among lipoproteins of cholesterol-fed African green and cynomolgus monkeys were studied. As TPC increased, the apo E distribution changed, with the majority of the apo E accumulating on large, cholesteryl ester–enriched plasma LDL particles. This accumulation in LDL appears to explain the high positive correlation between plasma TPC and apo E concentrations. Furthermore, based on the high positive correlation found between plasma apo E concentration and LDL apo E to apo B molar ratio, and the extent of coronary artery atherosclerosis, apo E enrichment of LDL appears to represent an atherogenic feature of diet-induced hypercholesterolemia.

Methods

Animals and Diets

Cynomolgus and African green monkeys from ongoing studies of dietary induction of atherosclerosis were used in this study. A group of 29 male cynomolgus monkeys were fed diets containing 40% of calories as either saturated fat (polyunsaturated saturated fatty acid ratio [P/S]=0.34) or polyunsaturated fat (P/S=2.2) and 0.3 mg cholesterol/kcal of diet. Two groups including 74 male African green monkeys (29 in group 1 and 45 in group 2) were fed diets with 0.8 mg cholesterol/kcal, with 40% of calories as either saturated (P/S=0.34) or polyunsaturated (P/S=2.2) fat. Five African green monkeys (group 3) were fed diets with 0.032 mg cholesterol/kcal and 40% of calories as saturated fat (P/S=0.34). The composition of plasma LDL was analyzed for all the cynomolgus monkeys (n=28) and African green monkeys of group 1 (n=29). Two subgroups of monkeys, including six cynomolgus monkeys and six African green monkeys, that were fed a saturated fat diet for about 5 years were selected to represent the spectrum of hypercholesterolemia and were killed for evaluation of the extent of coronary artery atherosclerosis.

Blood Collection

Animals were fasted for 18 hours and were restrained with ketamine hydrochloride (10 mg/kg body wt) before blood was drawn. Blood samples (20–30 ml) were drawn into plastic tubes containing Na2EDTA as an anticoagulant and antioxidant (final concentration, 1 mg/ml) and sodium azide as an antimicrobial agent (final concentration, 0.1 mg/ml Na3) and were then placed on ice. Plasma was obtained after low-speed centrifugation at 2,000 rpm for 30 minutes at 15ºC to separate plasma from red blood cells. Phenylmethylsulfonyl fluoride (PMSF) and aprotinin were added to plasma to achieve final concentrations of 80 µg/ml and 1.0 µg/ml, respectively. A 1.0-ml aliquot of plasma was frozen at −20ºC for up to 2 weeks for apolipoprotein and cholesterol measurements. The remaining plasma was used for lipoprotein fractionation.

Ultracentrifugation

Solid potassium bromide was added to 7 ml plasma to raise the density to 1.225 g/ml. The sample was placed in an ultracentrifugation tube and was over-layered with a d=1.225 g/ml KBr solution. Ultracentrifugation was carried out by using a Ti70.1 rotor at 50,000 rpm for 24 hours at 15ºC in a Beckman L5-50 ultracentrifuge (Beckman Instruments, Palo Alto, Calif.). Lipoproteins isolated in the d<1.225 g/ml supernatant (the upper 2–3 ml of the tube) were obtained by tube slicing. Aliquots of both the lipoprotein fraction (d<1.225 g/ml) and the nonlipoproteins...
tein fraction (d>1.225 g/ml) were frozen at -20°C for apolipoprotein and cholesterol measurements.

After ultracentrifugation, percentage recoveries of apo E, apo B-100, and cholesterol in the lipoprotein fraction and the nonlipoprotein fraction were as follows: apo E, 86.9±5.98% (mean±SEM, n=12); apo B-100, 72.2±6.62%; and cholesterol, 87.4±1.61%.

**Gel-Filtration Chromatography**

Lipoproteins were fractionated according to size from whole plasma or, in some cases, from the d<1.225 g/ml lipoprotein fraction by 4% agarose gel-filtration chromatography (Biogel A-15M, 200-400 mesh; Bio-Rad Labs, Richmond, Calif.; 1.6×100-cm glass column at 4°C) as described by Rudel et al.26 Whole plasma (7 ml) was concentrated by low-speed centrifugation in Centriflo CF-25 ultrafiltration cones (Amicon, Danvers, Mass.) to a final volume of 5 ml or less. Within 8 hours of blood collection, the whole-plasma concentrate was applied to a 4% agarose gel-filtration column equilibrated in 0.15 M NaCl, 0.01% EDTA, and 0.01% NaN3, pH 7.4. Lipoproteins in the d<1.225 g/ml fraction were then applied to the same 4% agarose gel-filtration column for separation according to size. In some experiments, the lipoproteins across the elution profile were pooled into six regions corresponding to VLDL, IDL (intermediate-size low density lipoproteins), LDL, the trough region (TR) between LDL and HDL, HDL, and region V (the region near V,, the total column volume). These regions were identified based on the elution profile at 280 nm of the d<1.225 g/ml lipoprotein fraction. Cholesterol, apo E, apo B-100, and apo A-I were quantified on the pooled column regions or on individual fractions across the elution profile.

Plasma LDL was isolated from 200 μl of the d<1.225 g/ml lipoprotein fraction by gel-filtration chromatography by using a Superose 6 HR-30 column (Pharmacia, Piscataway, N.J.). LDL composition, molecular weight, and apo B-100 and apo E content were determined from the isolated pooled plasma LDL. LDL molecular weight was calculated by the method of Rudel et al.26 by using a standard iodinated plasma LDL of known molecular weight.

**Equilibrium Density Ultracentrifugation**

Whole plasma from four saturated fat-fed African green monkeys of group 2 was fractionated by gel-filtration chromatography as described above, and 2 ml of the pooled LDL fraction was raised to a density of 1.080 g/ml with solid potassium bromide. Equilibrium density ultracentrifugation of LDL was carried out by use of a method similar to that of Marzetta and Rudel.28 Successive layers of solutions of defined density were placed in VTi50 rotor tubes by underlayering as follows: 13 ml of d=1.006 g/ml, 13 ml of d=1.019 g/ml, 11 ml of d=1.060 g/ml, and 2 ml of LDL at d=1.080 g/ml. Samples were centrifuged for 6 hours at 15°C at 50,000 rpm in a Beckman LS-50 ultracentrifuge. The rotor was stopped with the brake off, and the tubes were emptied from the top by pumping Flourinert (d=1.85 g/ml; 3M Co., West Haven, Conn.) into the bottom of the tube with an ISCO tube-draining apparatus (ISCO, Lincoln, Neb.). Approximately 1.2-ml fractions were collected, and apo B-100 and apo E were measured by enzyme-linked immunosorbent assay (ELISA) on fractions across the gradient. Blank tubes containing no sample were processed in an analogous manner to the sample tubes. To determine the density distribution, the refractive index of individual tubes was measured, and the density was determined for the salt mixture by using an empirically derived standard curve for the relation between refractive index and density.

**Apolipoprotein Analyses**

Apo E, apo B-100, and apo A-I were measured by an ELISA with a procedure that was essentially the same as that described for apo A-I by Koritnik and Rudel.29 In brief, 500 ng affinity-purified goat polyclonal antibody in 200 μl 0.01 M NaHCO3 buffer, pH 9.6, was coated per well into a 96-well polystyrene microtiter plate (Corning ELISA plate No. 25805-96, Corning, N.Y.), and the plate was then placed in a humid chamber overnight at 4°C. Unbound antibody was removed with three washes of 0.01 M phosphate-buffered saline (PBS) and 0.1% Tween-20 (pH 7.0). Serial dilutions of standardized plasma pools and samples were prepared in 0.01 M PBS, 0.1% Tween-20, 0.1% bovine serum albumin (BSA), and 0.01% NaN3 (pH 7.0), and these diluted standards and samples were then heated at 37°C (apo E and apo B-100) or 52°C (apo A-I) for 3 hours. A 200-μl aliquot of each standard or sample dilution was then placed in a microtiter plate well and allowed to incubate overnight at 4°C in a humid chamber. Unbound antigen was removed with washes as described above. A horseradish peroxidase-conjugated antibody preparation (the same polyclonal antibody used to coat the microtiter plate) was then added in 200 μl 0.01 M PBS, 0.1% BSA, and 0.1% Tween-20 (pH 7.0) and incubated overnight at 4°C in a humid chamber. The plates were again washed to remove unbound horseradish peroxidase-conjugated antibody. The microtiter plates were then developed with an enzymatic color reaction employing 0.01% o-dianisidine·2HCl (Sigma Chemical Co., St. Louis, Mo.) as the chromogen and 0.006% H2O2 (Fisher Scientific Co., Fair Lawn, N.J.) as the enzyme substrate in a 0.10 M sodium phosphate-citrate phosphate buffer, pH 5.0. A 200-μl aliquot of this solution was added to each well, and the reaction was allowed to proceed for 45 minutes at room temperature. The reaction was stopped by the addition of 25 μl of 2.0N HCl, and the optical density was then measured at 415 nm on a Dynatech MR 580 Microtiter plate reader (Dynatech, Chantilly, Va.).

Serial dilutions (three to six) of a control plasma pool were prepared, and each dilution was plated in duplicate. From these dilutions, an average apolipoprotein concentration value for a control plasma pool was calculated for each plate. Average concentration values from several plates were then averaged, and a between-
plate, or interassay, coefficient of variation was calculated by dividing the standard deviation by the mean concentration and multiplying by 100. A within-plate, or intra-assay, coefficient of variation was also calculated for each microtiter plate. Serial dilutions (three to six) of a control plasma pool were prepared, and each dilution was plated in duplicate. An average apolipoprotein concentration value for the control plasma pool was then calculated from these dilutions. From each of the three to six dilutions, a plasma concentration was calculated, and the average and the standard deviation of these values were used to calculate an intraplate coefficient of variation. Intraplate coefficients of variation from several microtiter plates were obtained, and the mean coefficient of variation was then calculated. The interassay and intra-assay coefficients of variation for apo E were 13.1% (nine plates) and 6.1% (nine plates), respectively; for apo B-100, 6.1% (17 plates) and 10.9% (12 plates), respectively; and for apo A-I, 9.3% (11 plates) and 7.5% (12 plates), respectively.

Chemical Compositions

Chemical compositions were determined on the LDL fraction collected from the chromatographic separation of lipoproteins in the d<1.225 g/ml fraction by using a Superose 6 HR 10/30 gel-filtration column (Pharmacia). Total cholesterol was measured with the o-phthalaldehyde method of Rudel and Morris30 or enzymatically31 (Boehringer Mannheim Diagnostics, Indianapolis, Ind.). Free cholesterol was also measured enzymatically32 (Wako Pure Chemical Industries, Ltd., Tokyo, Japan). Sterified cholesterol mass was calculated by subtraction of free cholesterol from total cholesterol mass and was then converted to cholesteryl ester mass by multiplying by 1.7 to correct for fatty acid mass. Values obtained in this manner were shown to agree (±7%, P>0.05, paired t test, n=6) with those obtained after extraction and thin-layer chromatographic separation procedures.33 Protein was determined by the method of Lowry et al34 with BSA, fraction V (Sigma), as the protein standard. Lipid phosphorus was measured by the method of Fiske and SubbaRow.35 Triglycerides were separated from other lipids by thin-layer chromatography on precoated silica gel 60 plates without fluorescent indicator (Merck, West Point, Pa.) with hexane/diethyl ether/glacial acetic acid, 70:30:1, vol/vol/vol, as the solvent system. Spots containing triglycerides were scraped from the plate and extracted with high-performance liquid chromatography-grade chloroform/methanol, 2:1, vol/vol. After extraction, triglyceride content was determined using the procedure of Sardesai and Manning.36 When triglyceride mass was too low to be detected by this assay, sample values were assumed to be less than 1.0% of total LDL mass, based on the limit of detection.

Immunoaffinity Chromatography

LDL fractions isolated from the agarose column (without prior centrifugation) were subjected to further purification on anti-apolipoprotein B immunoaffinity columns. These columns were prepared by crosslinking immunoaffinity-purified apo B antibodies (15 mg/ml Affi-gel) to Affi-gel 10 immunoaffinity medium purchased from Bio-Rad. The antibodies were isolated29 from antisera prepared in goats immunized with apo B-100 isolated from cynomolgus monkey LDL by the deoxycholate delipidation procedure of Helenius and Simons.37 The antisera were demonstrated to be monospecific for apo B by Western blotting38 and ELISA procedures against purified apo A-I, apo A-II, apo E, and apo-chylomicron by using chylomicrons isolated from monkey thoracic duct lymph.39 The anti-apolipoprotein B immunoaffinity columns had a binding capacity of 750 mg apo B; essentially no purified apo E eluted in the bound fraction from this column.

Coronary Artery Atherosclerosis Evaluations

For each species, six animals from the saturated fat-diet groups were selected for atherosclerosis evaluations. The range of TPC concentrations in each species was fully represented by the animals selected. This permitted correlation and regression analyses comparing lipoprotein, apolipoprotein, and atherosclerosis end points. At necropsy, hearts were perfusion-fixed for 1 hour at a pressure of 100 mm Hg with 10% neutral buffered formalin. Five 3-mm serial sections were trimmed from each of the three main coronary arteries and were dehydrated and embedded in paraffin. Slides were then prepared and stained with the Verhoeff-van Gieson stain. Coronary artery atherosclerosis was evaluated morphometrically as the mean intimal area (area between the internal elastic lamina and the lumen) for the 15 sections from each heart.40

Statistical Methods

All values represent the mean±SEM. Statistical comparisons were performed by paired t test. Most samples were normally distributed; however, logarithmic transformation was used before performing the t test on those parameters that were not normally distributed. Comparison of regression lines was performed as described by Snedecor and Cochran.41 Correlation and stepwise regression analyses were performed using the STATVIEW software (Abacus Concepts Inc., Berkeley, Calif.) for the Macintosh computer.

Results

The relation between TPC concentrations and whole-plasma apo E concentrations in cynomolgus and African green monkeys is shown in Figure 1. Whole-plasma apo E concentrations are significantly correlated to TPC concentrations (r=0.89, P<0.01) in both species of nonhuman primates. No species difference in this relation was found.

The concentration of plasma apo E in each lipoprotein region for selected low- and high-responding cynomolgus and African green monkeys is shown in Figure 2. For each species, six saturated-fat-fed animals with varying TPC concentrations were se-
selected. On average, the low-responding animals had an average TPC concentration of 174±24 mg/dl and were in the lower 20th percentile of TPC concentrations. The high-responding animals had an average TPC concentration of 398±18 mg/dl and were in the upper 40th percentile of TPC concentrations. The percentage distribution of apo E in pooled lipoprotein fractions was determined, and the concentration of plasma apo E in each lipoprotein column region was calculated. No significant differences between species in the percentage distribution of apo E was found by t test; therefore, the data for both species were combined for each response group (see Table 1). Plasma concentrations of apo E in the lipoprotein fractions are shown in Figure 2 as the averages (G.R. per milliliter, mean±SEM) of the six low- and six high-responding monkeys. While apo E was found in all lipoprotein regions including VLDL, IDLDL, LDL, and HDL for both the low- and high-responding animals, the concentration of plasma apo E was significantly different only in the LDL region of the high- versus the low-responding animals (72.9 versus 12.0 μg/ml, p<0.01). Correlation analysis was performed comparing lipoprotein cholesterol and apo E concentrations. Table 2 shows correlation coefficients for cholesterol and apo E in the LDL and HDL regions. The strong correlation found between TPC and plasma apo E concentrations, as shown in Figure 1, is consistent with the many significant correlations found here. In the groups of cynomolgus and African green monkeys studied, the dietary cholesterol-induced increase in TPC concentrations was highly correlated to increased LDL cholesterol concentrations (r=0.92, p<0.01). On the other hand, statistically significant relations were not found between HDL apo E and either TPC or plasma apo E concentrations, and no correlation was found between plasma apo E concentrations and plasma triglyceride concentrations (r=0.16, p=0.61) or the VLDL cholesterol of column region I (r=0.49, p=0.11). A more detailed analysis of the apo E, apo B-100, and apo A-I distribution among lipoprotein classes in individual animals revealed how the lipoprotein apo E distribution changed with the extent of hypercholesterolemia. Whole plasma from 11 animals with TPC concentrations ranging from 97.1 to 455 mg/dl was fractionated by 4% agarose gel-filtration chromatography. The cholesterol elution profiles for three African green monkeys selected to have widely different TPC concentrations of 122, 210, and 455 mg/dl are shown in Figures 3A, 3B, and 3C, respectively. This figure demonstrates the increase in LDL cholesterol relative to HDL cholesterol, from panels A to C, that has been reported to occur with the increasing degree of hypercholesterolemia in nonhuman primates fed dietary cholesterol.25 The elution profiles for whole-plasma apo E, apo B-100, and apo A-I for these three representative...
African green monkeys are shown in panels A, B, and C of Figure 4. This figure demonstrates several points. First, in plasma of the low-cholesterol animal (panel A), the majority of the plasma apo E eluted within the HDL region with apo A-I. The fact that the apo E peak was shifted to the left compared with apo A-I indicates that apo E was present on larger HDL particles. A minor proportion of the plasma apo E also eluted within the apo B-100 region. Virtually complete separation of the apo B-100 of LDL and the apo A-I of HDL was achieved in this animal and in all animals. Panel B demonstrates the apolipoprotein elution profiles from an animal with a moderate TPC concentration (210 mg/dl). Compared with that in panel A, a greater proportion of plasma apo E eluted with apo B-100. The proportion of apo E eluting together with apo A-I was approximately 50% lower in panel B compared with panel A. Panel C demonstrates the apolipoprotein elution profiles for an animal with a high TPC concentration (455 mg/dl). The majority of the apo E in plasma eluted within the apo B-100 region. The apo E peak was shifted to the left of the apo B-100 peak, indicating that the apo E eluted on larger lipoprotein particles that presumably also contained apo B-100. The apparent redistribution of the plasma apo E from the apo A-I to the apo B-100 region was primarily due to the increase in the amount of apo E eluting in the apo B-100 region; the amount in HDL remained nearly constant. Nine additional animals, seven African green monkeys (a TPC range of 129–454 mg/dl) and two cynomolgus monkeys (TPC concentrations of 97 and 419 mg/dl), were examined in the same fashion as shown in Figures 4 and 5. All data were consistent in demonstrating the pattern of apo E increase in the apo B-100 or LDL region and no change or a small decrease of apo E in the HDL region with increasing hypercholesterolemia.

LDL is the major cholesterol-containing lipoprotein in hypercholesterolemic cynomolgus and African green monkeys. To characterize the LDL particles from animals with varying degrees of hypercholesterolemia, plasma LDL from African green and cynomolgus monkeys were analyzed with respect to composition, size, and apo E content. The cholesteryl ester content and the apo E to apo B-100 molar ratio were higher in LDL with higher molecular weights. This point is clearly illustrated when individual-animal data are plotted, as in Figure 5. The data from both species and all diet groups fit the same regression line, showing that these relations are diet and species independent. The only exception to this trend was in the polyunsaturated fat–fed African green monkeys, for which no significant correlation was identified between LDL apo E to apo B-100 molar ratio and LDL molecular weight; however, these data appear to fit the line in Figure 5B. Taken together, these data suggest that, in general, LDL particles with more core cholesteryl ester had more surface apo E.

**Table 1.** Species Comparison of Apolipoprotein E Distribution Among Pooled Lipoprotein Column Regions for Fractionated Whole Plasma

<table>
<thead>
<tr>
<th>Animal + sample</th>
<th>VLDL</th>
<th>IDL</th>
<th>LDL</th>
<th>TR</th>
<th>HDL</th>
<th>V</th>
</tr>
</thead>
<tbody>
<tr>
<td>AG low (n=3)</td>
<td>0.570±0.24</td>
<td>11.37±3.77</td>
<td>33.00±12.8</td>
<td>5.100±1.23</td>
<td>47.60±11.9</td>
<td>2.360±0.40</td>
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<tr>
<td>C low (n=3)</td>
<td>0.071±0.02</td>
<td>14.90±5.05</td>
<td>37.80±11.6</td>
<td>7.460±1.19</td>
<td>32.30±12.4</td>
<td>7.520±3.19</td>
</tr>
<tr>
<td>Mean±SEM (n=6)</td>
<td>0.320±0.16</td>
<td>13.10±2.92</td>
<td>35.40±7.80</td>
<td>6.280±0.93</td>
<td>39.90±8.41</td>
<td>4.940±1.84</td>
</tr>
<tr>
<td>AG high (n=3)</td>
<td>1.840±0.72</td>
<td>11.00±3.54</td>
<td>79.80±5.39</td>
<td>7.270±0.85</td>
<td>4.280±1.63</td>
<td>0.420±0.11</td>
</tr>
<tr>
<td>C high (n=3)</td>
<td>0.097±0.07</td>
<td>10.70±1.75</td>
<td>67.30±4.92</td>
<td>11.60±2.70</td>
<td>9.05±2.72</td>
<td>1.410±0.59</td>
</tr>
<tr>
<td>Mean±SEM (n=6)</td>
<td>0.970±0.51</td>
<td>10.80±1.77</td>
<td>73.50±4.33</td>
<td>7.150±2.36</td>
<td>6.670±1.78</td>
<td>0.920±0.35</td>
</tr>
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</table>

All values are mean±SEM. Whole plasma was fractionated by gel-filtration chromatography, and lipoprotein regions were pooled as described in Methods. Within each response group, no statistically significant differences were found for the percentage distribution of apo E between AG vs. C. Therefore, the data were pooled.

Apo, apolipoprotein; VLDL, very low density lipoproteins; IDL, intermediate-sized low density lipoproteins; LDL, low density lipoproteins; TR, trough region; HDL, high density lipoproteins; V, region near V; AG, African green monkeys; C, cynomolgus monkeys; Clow, low; Chigh, high; AGlow, low; AGhigh, high. There were 12 monkeys in each comparison.

**Table 2.** Correlation Coefficients Between Apolipoprotein E and Cholesterol Concentrations of Low Density Lipoproteins and High Density Lipoproteins

<table>
<thead>
<tr>
<th></th>
<th>LDL apo E</th>
<th>HDL apo E</th>
<th>HDL apo E</th>
<th>HDL apo E</th>
<th>TPC</th>
</tr>
</thead>
<tbody>
<tr>
<td>LDL apo E</td>
<td>0.89*</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>HDL apo E</td>
<td></td>
<td>-0.77*</td>
<td>-0.72*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HDL apo E</td>
<td>-0.51*</td>
<td></td>
<td>-0.38</td>
<td>0.39</td>
<td></td>
</tr>
<tr>
<td>TPC</td>
<td>0.92*</td>
<td>0.78*</td>
<td>-0.69*</td>
<td>-0.49</td>
<td></td>
</tr>
<tr>
<td>Plasma apo E</td>
<td>0.98*</td>
<td>0.92*</td>
<td>-0.80*</td>
<td>-0.44</td>
<td>0.89*</td>
</tr>
</tbody>
</table>

There were 12 monkeys in each comparison.

LDL-C, low density lipoprotein cholesterol; HDL-C, high density lipoprotein cholesterol; LDL apo E, amount of apolipoprotein E associated with plasma low density lipoproteins; HDL apo E, amount of apolipoprotein E associated with plasma high density lipoproteins; TPC, total plasma cholesterol concentration.

*Significance at p≤0.01.
Figure 3. Comparison of lipoprotein cholesterol distribution among African green monkeys with increasing degrees of hypercholesterolemia. Whole plasma was fractionated by 4% agarose gel-filtration chromatography from three representative saturated fat-fed African green monkeys with varying degrees of hypercholesterolemia. Represented are animals with low (panel A), medium (panel B), and high (panel C) plasma cholesterol concentrations of 122, 210, and 455 mg/dl, respectively. Cholesterol was measured enzymatically as described in "Methods" and is expressed as micrograms total cholesterol per fraction; all fractions were 2.5 ml in volume. Every other fraction was measured, and each point represents one fraction.

Within the LDL density range (1.020–1.050 g/ml), Apo B-100 peaked in the 1.03–1.04 g/ml density range in all of these animals. Apo E was present in all apo B-100 particles across this gradient, although in each animal, the LDL particles in the 1.02–1.03 g/ml density range appeared to be apo-E enriched. The increase in apo E at the bottom of the gradient presumably resulted, at least in part, from the dissociation of apo E from lipoprotein particles by the gradient ultracentrifugation process.
Another method used to investigate the heterogeneity of the LDL population and to document the presence of apo E on apo B-containing particles was anti–apo B immunoaffinity chromatography. Six African green monkeys, selected to represent the range of plasma cholesterol concentrations, were studied. We collected the LDL fraction from the agarose column, passed an aliquot over the anti-apo B affinity column, and then quantified the amount of apo B and apo E in the bound and unbound fractions. The mean ± SEM apo E to apo B molar ratio in the starting LDL was 0.477 ± 0.06, and the mean cholesterol to apo B ratio for these particles was 2.36 ± 0.31. In the LDL subtraction bound to the anti-apo B column, the mean apo E to apo B molar ratio was 0.372 ± 0.05, a value that was not significantly different (p = 0.12) from that for the starting LDL. The mean cholesterol to apo B ratio in the bound fraction was 2.30 ± 0.13, a value that was not significantly different from that of the starting material. More than 60% of the apo B applied to the apo B column bound to the column, clearly establishing that most of the apo E in the LDL fraction was present on particles also containing apo B. On the other hand, the unbound material had an apo E to apo B molar ratio of 1.053 ± 0.2, and the cholesterol to apo B ratio for this material was 3.16. Thus, the presence of an apo E– and cholesterol-enriched subfraction of LDL particles was suggested.

Correlation and regression analyses were used to relate the lipoprotein and apolipoprotein measurements to the extent of coronary artery atherosclerosis in a subgroup of animals preselected to represent the range of plasma cholesterol concentrations in both species. The averages for the plasma lipoprotein and coronary artery atherosclerosis end points in these preselected groups are shown in Table 3. No species differences were found for most of the variables except for apo B, for which the average value for cynomolgus monkeys was higher than that for African green monkeys. Table 4 shows the correlation coefficients for these variables with coronary artery intimal area. Statistically significant correlation coefficients were seen for each measurement, with the highest single coefficient being between apo E and coronary artery intimal area, r = 0.82. The data in Figure 7 show the comparisons between intimal area and apo E and apo B. These apolipoprotein end points are highly correlated to the coronary artery atherosclerosis end point.

To better understand the importance of these measurements, stepwise regression analyses were done. The highest regression coefficient was $R^2 = 0.935$, which was obtained when apo B concentration and the LDL apo E to apo B molar ratio were entered into the equation. Apo E, TPC, LDL cholesterol, LDL molecular weight, and HDL cholesterol did not make significant contributions to the regression equation in stepwise regression analyses after apo B and the apo E to apo B molar ratio were entered into the equation. These two lipoprotein measurements explained a very large proportion (93.5%) of the variability in atherosclerosis in these animals, suggesting that the contribution of these measures to the development of athero-
sclerosis in these experimental primates was important. The other variables measured are highly intercorrelated and are also likely to contribute to the development of coronary artery atherosclerosis, but in this analysis, they did not contribute independently of apo B and the LDL apo E to apo B ratio.

Discussion

The current study demonstrates that plasma apo E concentrations were directly and positively correlated to TPC and LDL cholesterol concentrations in hypercholesterolemic African green and cynomolgus monkeys and to coronary artery intimal area, a measure of the extent of coronary artery atherosclerosis in the pri-

rosis. In hypercholesterolemic animals, the apo E found in plasma was primarily associated with LDL particles, and the degree of this association was higher when the LDL were more cholesteryl-ester enriched. The fact that we were able to demonstrate a high correlation between both apo E concentration and LDL apo E to apo B molar ratio and coronary artery intimal area lends credence to the hypothesis that association of apo E with LDL represents an atherogenic feature of diet-induced hypercholesterolemia. Correlation analyses do not prove cause and effect; however, the strength of the association is very high, and it seems likely that apo E–enriched LDL promote coronary artery atherosclerosis in the pri-

<table>
<thead>
<tr>
<th>Species</th>
<th>n</th>
<th>TPC (mg/dl)</th>
<th>LDL chol (mg/dl)</th>
<th>HDL chol (mg/dl)</th>
<th>LDL MW (g/mole)</th>
<th>LDL apo E/apo B (mole/mole)</th>
<th>Apo B (mg/dl)</th>
<th>Apo E (mg/dl)</th>
<th>Coronary artery intimal area* (mm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>African green</td>
<td>6</td>
<td>346±60</td>
<td>236±67</td>
<td>94±10</td>
<td>3.43±0.28</td>
<td>0.53±0.20</td>
<td>97±16</td>
<td>8.6±2.5</td>
<td>1.509±0.549</td>
</tr>
<tr>
<td>Cynomolgus</td>
<td>6</td>
<td>322±58</td>
<td>221±51</td>
<td>66±9</td>
<td>3.41±0.18</td>
<td>0.38±0.13</td>
<td>183±26</td>
<td>10.5±2.2</td>
<td>2.45±0.45</td>
</tr>
<tr>
<td>Probability</td>
<td>0.79</td>
<td>0.85</td>
<td>0.06</td>
<td>0.95</td>
<td>0.52</td>
<td>0.02</td>
<td>0.59</td>
<td>0.22</td>
<td></td>
</tr>
</tbody>
</table>

All values are mean±SEM.

TPC, total plasma cholesterol; LDL, low density lipoproteins; Chol, cholesterol; HDL, high density lipoproteins; MW, molecular weight; apo, apolipoprotein.

*Coronary artery intimal areas were logarithmically transformed to normalize the distribution.

†As determined by two-tailed, unpaired t test to compare species.

‡df = 5.
TABLE 4. Coronary Artery Atherosclerosis—Lipoprotein Relations

<table>
<thead>
<tr>
<th>Log of coronary artery intimal area vs.</th>
<th>n</th>
<th>r</th>
</tr>
</thead>
<tbody>
<tr>
<td>Apo E</td>
<td>12</td>
<td>0.82</td>
</tr>
<tr>
<td>TPC</td>
<td>12</td>
<td>0.80</td>
</tr>
<tr>
<td>LDL chol</td>
<td>12</td>
<td>0.80</td>
</tr>
<tr>
<td>Apo B</td>
<td>12</td>
<td>0.78</td>
</tr>
<tr>
<td>LDL MW</td>
<td>12</td>
<td>0.73</td>
</tr>
<tr>
<td>LDL apo E/apo B</td>
<td>11</td>
<td>0.63</td>
</tr>
<tr>
<td>HDL chol</td>
<td>12</td>
<td>-0.57</td>
</tr>
</tbody>
</table>

Apo, apolipoprotein; TPC, total plasma cholesterol; LDL, low density lipoproteins; Chol, cholesterol; MW, molecular weight; HDL, high density lipoproteins.

While the concept that apo E may promote atherosclerosis development is not new, we believe that this study is the first to directly quantify the extent of this association.

We have consistently found significant individual variability in the degree of hypercholesterolemia induced by atherogenic diets in nonhuman primates.25–28 In each case, LDL were the primary lipoprotein to accumulate as plasma cholesterol concentrations increased. The correlation reported in the current study was significant over the range of plasma cholesterol values of approximately 100–700 mg/dl. In contrast, in normal human subjects apo E and plasma cholesterol concentrations (r=0.35) were not highly correlated, whereas plasma triglyceride concentrations were better correlated to apo E (r=0.65) and were found to be better predictors of plasma apo E concentrations.19 This may indicate that apo E has a preference for associating with triglyceride-rich lipoproteins, as it does in human subjects. The average plasma triglyceride concentration of the monkeys in the present study was 20 mg/dl. Thus, triglyceride-rich lipoproteins are not present in appreciable amounts and would not cor-

Figure 7. Scatterplots showing correlation between coronary artery atherosclerosis extent and plasma apolipoprotein (apo) B (panel A) and apo E (panel B) concentrations. Six male cynomolgus monkeys (•) and six male African green monkeys (□) were fed an atherogenic diet containing saturated fat for 5–6 years (see “Methods”). During this time, plasma samples were collected and apolipoproteins were measured annually by ELISA for apo B and apo E. Mean values for each animal were calculated and correlated with the extent of coronary artery atherosclerosis as measured by the average coronary artery intimal area (see “Methods”). Line of best fit is shown. ELISA, enzyme-linked immunosorbent assay.
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Varying degrees of hypercholesterolemia were correlated with plasma apo E concentrations. Perhaps if these types of lipoproteins had been present in greater amounts, less apo E would have been associated with LDL and the rate of development of atherosclerosis would have been less. This possibility assumes that accumulation of cholesteryl ester–rich LDL in the artery is more likely to promote atherosclerosis than is accumulation of a triglyceride-rich lipoprotein such as VLDL.

A quantitative approach was used to evaluate the distribution of apo E among plasma lipoproteins in relation to the plasma lipoprotein distribution. Lipoproteins were separated by size, without ultracentrifugation, and the distribution of apo E among the plasma lipoproteins was compared with that of apo B-100 and apo A-I. A systematic shift in the distribution of apo E from a predominance in the apo A-I (HDL) region of the column to the apo B-100 (LDL) region of the column was noted when the apolipoprotein distributions among several animals with varying degrees of hypercholesterolemia were compared. When column regions were pooled so that the concentration of plasma apo E in each fraction could be determined, it was found that the concentration of apo E in the apo A-I (HDL) region of the column did not change significantly. Rather, the shift in the distribution of apo E was due to a real increase in the concentration of apo E in the apo B-100 (LDL) region. Therefore, the change in plasma apo E concentration was due almost exclusively to an accumulation of apo E on LDL particles.

Such accumulation of apo E on LDL has not been previously reported. Melchoir et al studied cholesterol-fed rhesus monkeys and reported a positive correlation between plasma apo E and cholesterol concentrations, but these investigators stated that the accumulation of apo E was on IDL, not LDL. The current study clearly demonstrates that apo E accumulated primarily on LDL particles, as defined by size and density. The reasons for the different conclusion between the two studies may, in part, be a result of the different density gradient procedures used to define IDL versus LDL. Other methodologic factors also may contribute to this difference, such as the different apo E quantification procedures used (electroimmunoassay versus ELISA). However, the most significant contributing factor would appear to be the differing levels of hypercholesterolemia between the animals in the two studies. The hyperresponding rhesus monkeys had plasma cholesterol concentrations approximately two times higher than the monkeys of the present study (800 versus 400 mg/dl, on average). The higher levels of plasma cholesterol are known to be associated with increased levels of IDL and β-VLDL. These apo E–enriched lipoproteins may also promote atherosclerosis, although atherosclerosis was not an end point measured in the rhesus monkey study. The majority of atherosclerosis that occurs in humans does not occur at plasma cholesterol levels near 800 mg/dl, thus, the relevance of our studies at more modest levels of hypercholesterolemia.

Studies of hypercholesterolemia in humans and other animal models have described accumulation of apo E primarily in VLDL and HDL. The accumulation of apo E in HDL occurred in an unusual particle called HDLc, and this lipoprotein is not typically found in physiological concentrations among HDL in most animals and has not been identified in our studies of monkeys. The highest concentrations of HDLc have been found in animal species that have low levels of cholesteryl ester transfer activity. Our animals have been found to have high concentrations of cholesteryl ester transfer protein that themselves were positively correlated with coronary artery atherosclerosis.

A paradox occurs in the current studies when one considers that apo E was found to accumulate on cholesteryl ester–rich LDL particles and that LDL turnover time was found to be positively correlated with average LDL size. Apo E has been shown to be a high-affinity ligand for the LDL receptor, and apo E is also apparently a ligand for LRP. Therefore, it is paradoxical that lipoprotein particles enriched in apo E accumulate in plasma, as one would expect that apo E enrichment of the lipoprotein would result in more rapid clearance from plasma.

Several possibilities may help explain this paradox. Kowal et al have shown that in vitro enrichment of β-VLDL with exogenous apo E is necessary for interaction with the LRP. Thus, it appears that much of the endogenous apo E on the particle is not in a receptor-active form. In addition, binding to the LDL receptor may also require a receptor-active form, and this may not be available on the LDL particles found in our cholesterol-fed monkeys. However, St. Clair et al have shown that LDL isolated from hypercholesterolemic cynomolgus monkeys interacted with the LDL receptor of skin fibroblasts with a greater binding affinity compared with LDL isolated from normocholesterolemic animals. This could indicate that the apo E that accumulates on plasma LDL of cholesterol-fed monkeys may interact with the LDL receptor. However, the binding of LDL to LDL receptors in these animals would be limited because this receptor is downregulated by dietary cholesterol. In this case, the decreased rate of LDL catabolism in vivo due to the downregulation of the LDL receptor may help explain why apo E–rich LDL particles accumulate in the plasma of these animals.

The balance between production and catabolism determines plasma LDL concentration. African green monkey liver perfusion studies have shown that rates of hepatic apo B-100 and apo E accumulation in the perfusate were equivalent among animals with widely different plasma LDL cholesterol concentrations. A variety of apo B-100 lipoproteins were secreted during liver perfusion, with the characteristics of VLDL, IDL, and LDL. Their metabolism to plasma LDL has been examined in vivo. All of
these apo B-100 lipoproteins apparently have the potential to form plasma LDL, although the factors determining which particles become LDL are unknown. One possible factor may be related to the presence or absence of apo E. Yamada et al.8-10 using apo E immunofinity chromatography, have shown that the majority of apo B-100 lipoproteins secreted by the perfused rabbit liver contain apo E (75-80%), although a population of apo B-100 lipoproteins deficient in apo E was also present. This may also apply to the monkeys of the present studies, as we have found that the majority of apo B-100 perfusate lipoproteins were apo E enriched.49

Given the similar production rates between groups of normal and hypercholesterolemic WHHL rabbits, Yamada et al8-10 have developed a kinetic model for LDL production that takes into account apo E heterogeneity among lipoprotein particles, although this model assumes that LDL are produced entirely from VLDL. This is most likely not a valid assumption for nonhuman primates because a variety of apo B-100 lipoproteins are found during liver perfusion. In normocholesterolemic animals, apo E-enriched VLDL, IDL, and LDL would have an increased rate of plasma clearance due to the presence of apo E and would not be converted to plasma LDL particles. The apo B-100-only lipoproteins would not be cleared to a significant extent by the LDL receptor, and increased conversion to plasma LDL particles could result. However, in situations in which the receptor is downregulated, such as in WHHL rabbits and hypercholesterolemic nonhuman primates, the hypothesis predicts that apo E-enriched apo B-100 lipoproteins would not be efficiently removed by the LDL receptor. As a result, precursor particles would be more efficiently metabolized into plasma LDL. In this case, apo E-enriched LDL particles could also accumulate in plasma, and plasma LDL concentrations would become elevated due to overproduction as a result of a decreased rate of catabolism of LDL precursor particles. The findings of increased LDL apo E to apo B-100 molar ratio, particle size, and elevated TPC and LDL cholesterol concentrations, as found in hypercholesterolemic nonhuman primates, are consistent with this possibility.

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KEY WORDS • apolipoprotein B • diet-induced atherosclerosis • low density lipoprotein heterogeneity • cholesterol-fed monkeys
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S C Stevenson, J K Sawyer and L L Rudel

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