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Research Article

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Phenotype-dependent Differences in Apolipoprotein E Metabolism and in Cholesterol Homeostasis in Human Monocyte-derived Macrophages

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Abstract

In this study, we investigated the impact of the common apoE polymorphism on apoE metabolism and cholesterol homeostasis in monocyte-derived macrophages isolated from E2/2, E3/3, and E4/4 subjects. Unloaded cells of all genotypes contained similar amounts of free cholesterol, cholesteryl ester, and apoE mRNA. E3/3 cells secreted 77 and 30% more apoE than E2/2 or E4/4 cells, respectively. Pulse-chase studies confirmed that the apoE secretion rate was greatest in E3/3 and least in E2/2 cells and showed that a portion of apoE2, but not apoE3 or apoE4, was degraded intracellularly. Surface binding of apoE was greatest in E4/4 cells, as revealed by heparinase treatment. On cholesterol loading with acetylated LDL, apoE mRNA levels and protein secretion rose most in E4/4 and least in E2/2 cells. Cholesterol and cholesteryl ester content, however, rose most in E2/2 and least in E3/3 cells. Incubations with ^{3}H-cholesterol–labeled acetylated LDL revealed that E2/2 cells were most efficient at secreting cholesterol. The greatest reuptake of ^{3}H-cholesterol–rich particles was from E4/4 macrophage–conditioned media. Thus, E2/2 macrophages, despite a low apoE secretion rate, are protected from cholesterol storage by apoE-mediated cholesterol efflux. In E3/3 macrophages, cholesterol accumulation is lessened by a high basal apoE secretion rate. E4/4 macrophages secrete the most apoE but lack effective net cholesterol efflux due to enhanced surface binding and reuptake of cholesterol-rich particles. (*J. Clin. Invest.* 1998. 101:1670–1677.) Key words: apoE polymorphism • cholesterol efflux • atherosclerosis • human monocyte–derived macrophages • Alzheimer's disease

Introduction

Three common isoforms of apoE exist in humans. These differ in only two amino acid positions and are designated E2, which contains cysteine at residues 112 and 158, E3 with cysteine at 112 and arginine at 158, and E4 with arginine at both 112 and 158 (1). These isoforms are coded for by the respective alleles termed ε2, with a frequency in Caucasian populations of 8%, ε3 with a frequency of 77%, and ε4 with a frequency of 15% (2). This polymorphism affects the risk of both coronary atherosclerosis (CHD)[1] and Alzheimer's disease, both of which are more common in persons expressing the E4 isoform (3, 4).

The isoforms differ from each other in their binding affinity for the LDL and other apoE receptors (5). Thus the apoE polymorphism has a significant impact on plasma lipoprotein levels. Total and LDL cholesterol levels are greatest in persons carrying the apoE4 isoform, intermediate in those with the apoE3 isoform, and lowest in those with the apoE2 isoform (6–9). Traditionally, the polymorphism-associated variation in CHD risk has been attributed to this effect on lipoproteins. However, phenotype-specific differences have also been shown in the ability of apoE to promote cholesterol efflux from cultured cells (10).

Although the bulk of circulating apoE is produced in the liver, significant amounts are also produced by macrophages, including those within the atherosclerotic plaque (11, 12). Several recent studies in transgenic mice have clearly illustrated the ability of macrophage-produced apoE to protect from atherosclerosis (13–16). In studies by Shimano et al. (15) and Bellosta et al. (16), a human apoE transgene was expressed specifically by macrophages in the arterial wall of mouse strains predisposed to development of diet-induced atherosclerosis. In both studies, this expression protected the animals from atherosclerosis, even in the presence of an atherogenic profile of circulating lipoproteins. It is likely that this protective effect of apoE is due at least in part to the apoE-mediated efflux of cholesterol from macrophages (17–19).

Therefore, it is possible that the differences in CHD risk among carriers of the various apoE phenotypes are also related to phenotype-specific differences in apoE production by macrophages. The aim of this study was to assess the impact of the apoE polymorphism on apoE gene expression and protein secretion, as well as on cell cholesterol accumulation, in an in vitro model of foam cell development, the cholesterol-loaded human monocyte–derived macrophage.

Methods

Subjects. All experiments described in this report were performed on cells from homozygous donors. Donors were identified either through the lipid clinic at our hospital or from medical students or colleagues who underwent a screening examination. The apoE polymorphism was determined by isoelectric focusing of delipidated serum and subsequent anti–apoE-immunoblotting as described previously (20). The characteristics of the donors are shown in Table I. All donors gave informed written consent. The procedure for monocyte isolation was approved by the Hospital Ethics Committee.

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1. *Abbreviations used in this paper:* AcLDL, acetylated LDL; CHD, coronary heart disease; RT, reverse transcriptase.

Table I. Plasma Lipid Parameters of the Subjects at Time of Isolation of Monocytes by Monocytapheresis

Phenotype	Age (yr)	*n*, Sex	Cholesterol	Triglycerides	LDL-C	HDL-C
				mg/dl		
E2/2	50±7	6 m	254±40	264±66	159±33	43±5
E3/3	29±1	5 m, 1 f	219±16	106±41	140±16	57±9
E4/4	40±18	3 m, 3 f	215±42	213±81	116±45	56±19

Data are expressed as mean±SD. *m*, male; *f*, female; *LDL-C*, LDL-cholesterol; *HDL-C*, HDL-cholesterol.

Cell culture. Monocytes were isolated by elutriation and countercurrent centrifugation as described previously (21). Purity of isolated monocytes was > 95% as revealed by FACS® analysis. Monocytes were maintained in RPMI 1640 medium (GIBCO, Eggenstein, Germany) supplemented with 20% pooled human serum for 14 d to allow differentiation into macrophages. Thereafter, cells were washed three times with serum-free RPMI 1640 and incubated under the conditions indicated in each section.

Lipoprotein isolation and labeling. LDL was obtained from human plasma from healthy volunteer donors by sequential ultracentrifugation (d = 1.019–1.063). Acetylated LDL (AcLDL) was obtained by repeated additions of acetic anhydride as previously described by Basu et al. (22) and radiolabeled by incubation with 200 μCi ^{3}H-cholesteryl-oleate (NEN-Du Pont, Bad Homburg, Germany) per milliliter AcLDL in the presence of lipoprotein-deficient serum for 6 h at 37°C (23). The reaction mixture was then adjusted to density 1.063 g/ml and ultracentrifuged for 2.5 h at 4°C (TL100; Beckman Instruments, Fullerton, CA) in 1.5-ml polypropylene tubes. Top fractions were pooled and thoroughly dialyzed in 0.9% NaCl. The product was analyzed by agarose gel electrophoresis to confirm the mobility shift of AcLDL versus normal LDL. Protein content was measured by the method of Lowry (24).

Quantitative reverse transcription (RT)–PCR. Macrophages were incubated in the presence or absence of 80 μg/ml AcLDL, or in RPMI 1640 only, for 48 h and washed twice with sterile PBS. RNA was obtained by guanidinium-phenol-chloroform extraction (25). Quantitative RT-PCR was performed as described by Grassi et al. (26) with slight modifications. Briefly, an internal RNA standard differing from wild-type apoE mRNA only by virtue of a 20-base insertion in the middle was prepared by sequential PCR reactions and transcription by T7-RNA polymerase. Its concentration was evaluated by densitometric analysis by comparison with known amounts of standard RNA. Increasing amounts of internal standard were added to 0.2-μg aliquots of cell RNA before the RT step. After denaturing for 3 min at 94°C, 30 cycles of PCR were performed at 94°C for 30 s, 58°C for 30 s, and 72°C for 45 s, followed by a final 5-min extension at 72°C. The primers used for this step were: forward primer: 5′-aag gac gtc ctt ccc cag gag c-3′, reverse primer: 5′-ctt cat ggt ctc gtc cat cag c-3′. The bands corresponding to wild-type RNA (308 bp) and internal standard (328 bp) were analyzed by densitometric scanning. The internal standard/wild-type RNA band intensity ratio was plotted against the number of molecules of internal standard that had been added. The concentration of wild-type apoE mRNA was read from the point on the regression line at which the ratio of internal standard/wild-type RNA was equal to one. In preliminary studies, this assay showed a coefficient of variation of 13%.

Measurements of apoE secretion. Macrophages were incubated in the presence or absence of 80 μg AcLDL for 48 h, washed and cultivated for a further 24 h in serum-free RPMI 1640 medium. This medium was collected, centrifuged to remove cell debris, and analyzed for apoE secretion by sandwich ELISA as described previously (10). In some cases, cells were incubated for 1 h at 4°C in the presence of 10 mM suramin (Calbiochem, Bad Soden, Germany) or 4 U/ml heparinase (Sigma, Deisenhofen, Germany) to assess the amount of apoE bound to the cell surface (27).

Quantification of synthesis, secretion, and degradation of apoE. A pulse-chase design was used to investigate the production, secretion, and degradation rate of apoE in unloaded macrophages. Cells were washed three times with methionine-free RPMI 1640 and incubated for the indicated time in the same medium in the presence of 100 μCi/ml ^{35}S-methionine (NEN-DuPont). After this pulse incubation, the medium was collected and stored at −70°C until analysis. Cells were washed three times with PBS/2 mM EDTA and harvested in 1 ml of lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, Protease Inhibitor Cocktail). A portion of the cells that had been pulsed for 40 min was used for multiple chase incubations in RPMI 1640 medium containing 500 μM unlabeled methionine. Thereafter the media were removed and the cells washed and harvested as described above. For immunoprecipitation of apoE, the same number of TCA-precipitable counts was used from each cell or medium sample. Samples were incubated first with anti-apoE antiserum (WAK Chemie, Bad Homburg, Germany) and then with protein G agarose beads (Boehringer Mannheim, Mannheim, Germany) as recommended by the manufacturer of the immunoprecipitation kit (Boehringer Mannheim). The precipitated material was denatured at 95°C for 3 min and separated by 13% SDS-PAGE. After electrophoresis, gels were exposed to a BAS-IIIs membrane suitable for bioimaging (BAS1500 Bio-Imaging System; Fujifilm, Tokyo, Japan) to quantify apoE by densitometric scanning. The proteins of the gels were then electroblotted to a nitrocellulose sheet to verify the identity of the immunoprecipitated protein as apoE by means of immunodetection.

Cellular cholesterol measurement. Macrophages were loaded with cholesterol by incubation with 80 μg/ml AcLDL (protein content) in RPMI 1640 for 48 h. Control macrophages were incubated in RPMI 1640 alone for the same period of time. Cells were then washed three times with serum-free medium and equilibrated in the same medium for a further 24 h. After extensive washing with PBS, cells were harvested in 2 ml distilled water and analyzed for cholesterol and cholesteryl ester accumulation by HPLC, as described previously (28).

Uptake and secretion of ^{3}H-cholesterol by cells. To investigate whether differences in apoE production by the various macrophage phenotypes were related to differences in the secretion and/or reuptake of cholesterol-rich particles, cells of each phenotype were incubated with 200 μg/ml ^{3}H-AcLDL for 8 h, washed three times with serum-free medium, and equilibrated in the same medium overnight. Conditioned media were then added to fresh E3/3 cells for 24 h. Cells and media were collected after each incubation step and analyzed for cholesterol content by TLC. Samples were delipidated with chloroform/methanol, 1:2, dried down, and redissolved in 20 μl chloroform. 2 μl were applied to Kieselgel HP 60 TLC plates (Merck, Darmstadt, Germany) which were eluted in hexane/heptane/diethylether/acetic acid, 63:18.5:18.5:1 vol/vol (29). The spots corresponding to free and esterified cholesterol were scraped off the plates and analyzed by scintillation counting (LKB, Uppsala, Sweden).

Statistics. Where appropriate, statistical analyses were performed using nonparametric Kruskal-Wallis one-way ANOVA for comparison among the three phenotypes. Comparison between individual phenotypes was by means of a Mann-Whitney U test. For paired samples, the Wilcoxon matched-pairs ranked-signs test was used (30). Multiple test situations were not taken into account. P values of ≤ 0.05 were taken to be significant.

Results

ApoE mRNA levels in macrophages. ApoE mRNA was present at the same level in unloaded cells of all three phenotypes (2.6×10^8 copies/μg cell RNA; Fig. 1), as measured by compet-

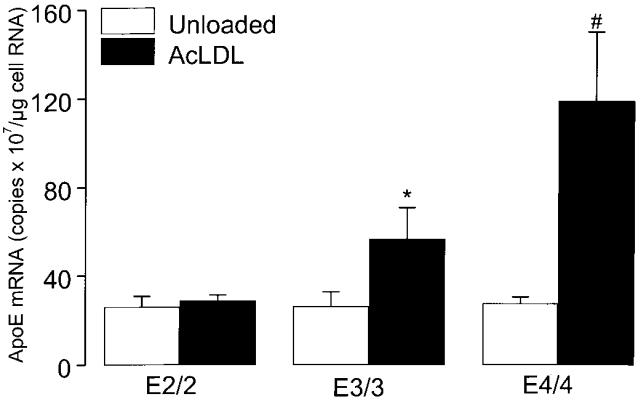

Figure 1. ApoE mRNA levels in unloaded and cholesterol-loaded human monocyte–derived macrophages. Cells of five donors per phenotype were incubated in serum-free RPMI 1640 or with 80 μg/ml AcLDL for 48h, washed with sterile PBS and harvested in guanidinium isothiocyanate for RNA extraction. ApoE mRNA levels were determined by competitive RT-PCR as described in the Methods. Results are expressed as mean±SEM of three to seven determinations per donor and loading condition. The differences among the three phenotypes in AcLDL-loaded cells were statistically significant at the $P < 0.05$ level (ANOVA); *$P < 0.05$ versus E2/2 and E4/4; #$P < 0.01$ versus E2/2. On cholesterol loading, apoE mRNA increased significantly in the E3/3 ($P < 0.05$) and E4/4 ($P < 0.01$), but not in the E2/2 cells.

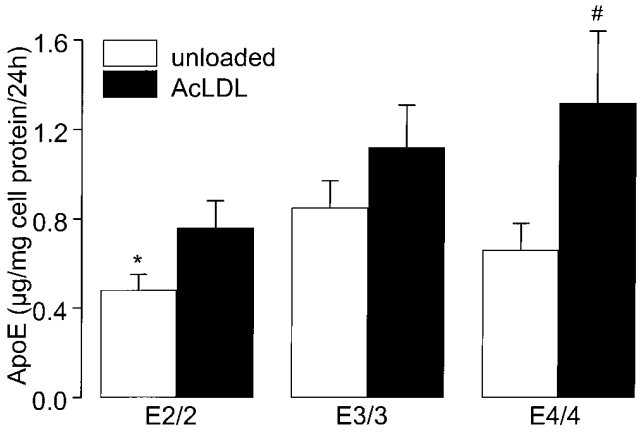

Figure 2. Secretion of apoE into the cell culture medium by control and cholesterol-loaded human monocyte-derived macrophages. Cells of six donors per phenotype were incubated in serum-free RPMI 1640 (*unloaded*) or with 80 μg/ml AcLDL for 48 h, washed and incubated in serum-free RPMI 1640 for a further 24 h. ApoE levels in the medium were determined by sandwich ELISA. Results are expressed as mean±SEM of three to seven determinations per donor and loading condition. The differences among the three phenotypes in AcLDL-loaded cells were statistically significant ($P < 0.05$); *$P < 0.05$ versus E3/3; #$P < 0.05$ versus E2/2. On cholesterol loading, apoE secretion increased significantly in all three phenotypes (E2/2 and E3/3 $P < 0.05$, E4/4 $P < 0.01$).

itive RT-PCR. On cholesterol loading, significant increases occurred in the E4/4 (4.3-fold, $P < 0.01$) and, to a lesser extent (2.2-fold, $P < 0.05$), in the E3/3, but not in the E2/2 cells (Fig. 1). The difference in the increase in apoE mRNA between the phenotypes was also significant ($P < 0.05$).

Secretion of apoE into macrophage culture medium. In the unloaded state, E3/3 cells secreted a net amount of 0.85±0.12 μg apoE/mg cell protein per 24 h (mean±SEM) into the culture medium (Fig. 2). Despite containing the same amount of apoE mRNA as cells of the other two phenotypes, E3/3 cells secreted 77 and 30% more apoE than E2/2 and E4/4 cells, respectively (E2/2 versus E3/3, $P < 0.05$, E3/3 versus E4/4 NS). In the loaded state, significantly more apoE was secreted by all three phenotypes as compared with the unloaded state (loaded versus unloaded E2/2 and E3/3 $P < 0.05$, E4/4 $P < 0.01$, Fig. 2). ApoE secretion in the loaded state was in the order E4/4 > E3/3 > E2/2, paralleling the mRNA levels in these cells (E4/4: 1.32±0.24, E3/3: 1.12±0.19, E2/2: 0.76±0.12 μg apoE/mg cell protein per 24 h). The secretion of apoE of the three phenotypes in the loaded state differed significantly ($P < 0.05$). It is of note that the significant twofold increase in net apoE secretion into the medium on cholesterol loading in E2/2 cells occurred in the presence of a constant level of apoE mRNA.

Quantification of synthesis and secretion rates of apoE. Previous work has shown that apoE may undergo intracellular degradation and thus escape secretion (31, 32). To investigate this possibility as the basis for the phenotype-related difference in apoE secretion, we measured the rate of apoE synthesis in unloaded macrophages by means of pulse-chase experiments. The incorporation of ^{35}S-methionine into cell-associated apoE after shorter pulse incubations appeared to be lower in E2/2 than in E3/3 or E4/4 cells (Table II). The secretion of ^{35}S-apoE into the pulse medium was in the order E3/3 > E4/4 > E2/2 (Table II), in agreement also with the ELISA measurements after 24 h (Fig. 2). Since apoE mRNA levels in the three phe-

Table II. Quantification of Synthesis of ApoE by Immunoprecipitation after Pulse Labeling with ^{35}S-methionine

	E2/2		E3/3		E4/4	
Duration of pulse (min)	Cells	Medium	Cells	Medium	Cells	Medium
40	5.3±0.8	1.1±0.4	15.0±2.0	5.2±3.0	10.7±4.0	1.8±0.6
60	7.2±2.8	2.8±1.0	17.5±4.5	8.3±1.7	14.0±2.0	6.8±2.5
240	21.2±6.2	9.0±3.8	19.5±4.5	33.8±0.9	14.4±0.4	13.0±3.1

Cells of four donors per phenotype were incubated in methionine-free RPMI 1640 containing ^{35}S-methionine (100 μCi/ml) for the indicated times. After incubations, cell samples were lysed and media centrifuged. TCA-precipitable counts were assessed for each sample. An equal count number was used for immunoprecipitation of apoE. Final pellets were separated by SDS-PAGE and gels were exposed on a membrane specific for bioimaging analysis. ApoE bands were scanned and densitometric values were converted into cpm units by comparison with a standard curve coexposed with the gel. Values are expressed as cpm apoE per cpm total labeled protein ($\times 10^{-5}$, mean±SEM of two to three measurements per donor).

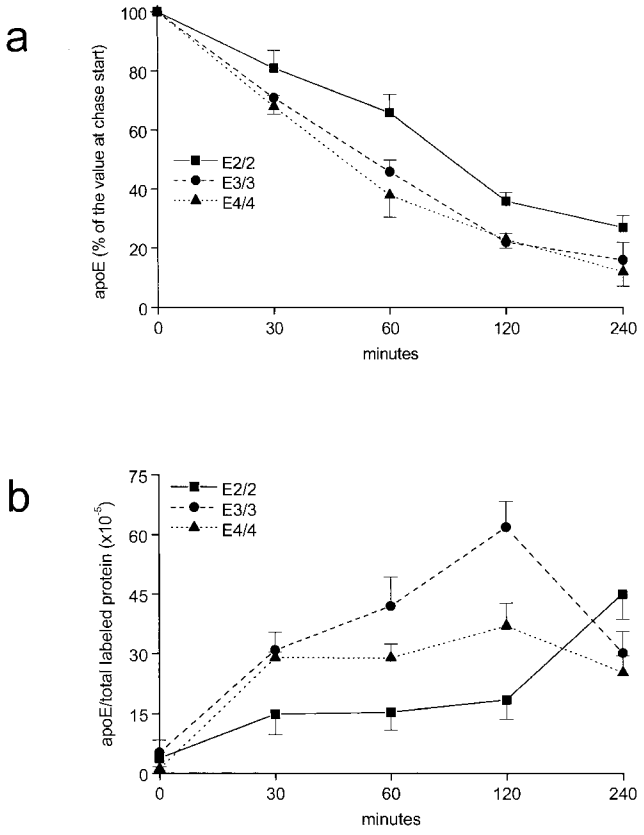

Figure 3. Turnover of ^{35}S-apoE in noncholesterol loaded human monocyte–derived macrophages. (*a*) Percentage of ^{35}S-apoE remaining in the cell after the times shown as a percentage of the level of ^{35}S-apoE at the end of a pulse incubation in methionine-free RPMI 1640 medium containing ^{35}S-methionine (100 μCi/ml) for 40 min (values at this time are shown in Table II). (*b*) Appearance of ^{35}S-apoE in the medium at the times shown after the end of 40 min pulse incubation. Cells of four donors per phenotype were investigated. After incubation, cells were harvested and lysed, and media were collected and centrifuged. TCA-precipitable counts were assessed for each sample. An equal count number was used for immunoprecipitation of apoE from cell or media samples. Final pellets were separated by SDS-PAGE and gels exposed on a membrane suitable for bioimaging analysis. ApoE bands were scanned and densitometric values were converted into cpm units by comparison with a standard curve coexposed with the gel. Results are expressed as mean±SEM of two to three measurements per donor.

notypes were similar, it would appear that the greatest degradation of newly synthesized apoE occurred in the E2/2 cells. The time course of ^{35}S-apoE metabolism, as measured by chase incubations at various times, showed that apoE2 disappeared more slowly from the cells than apoE3 or apoE4 (Fig. 3 *a*). The secretion rate of ^{35}S-apoE into the medium, as expected, was in the order E3/3 > E4/4 > E2/2 (Fig. 3 *b*). However, apoE3 and apoE4 levels reached a peak at 120 min and then declined, whereas apoE2 accumulated in the culture medium throughout the experimental period, possibly as a result of impaired reuptake of newly synthesized apoE by the E2/2 cells (Fig. 3).

Release of surface-bound apoE from macrophages by suramin or heparinase. At 4°C active secretion of apoE does not occur, so that only apoE that was bound to the cell surface appears in the medium. Under control conditions, apoE was released from cells in the order E4/4 > E3/3 > E2/2 (Table III). The additional amount of apoE released by suramin was similar for all three phenotypes (5.2–7.6 ng/mg cell protein). Heparinase, by contrast, released more apoE from E4/4 cells (11.4 ng/mg cell protein) than from E2/2 or E3/3 cells (7.5 and 1.5 ng/mg cell protein, respectively).

Table III. Displacement of Bound apoE from the Surface of Human Monocyte–derived Macrophages by Suramin and Heparinase

Treatment	E2/2	E3/3	E4/4
Control	9.5±0.5	14.2±1.5	24.6±8.6
Suramin 10 mM	17.1±1.0	19.4±3.5	31.4±9.7
Heparinase 4 U/ml	16.0±2.2	15.7±3.9	36.0±7.5

After 14 d in culture, macrophages isolated from three donors per phenotype were washed three times with serum-free RPMI 1640 medium on ice and incubated in the same medium for 1 h at 4°C in the presence of the indicated agents. Media were then collected and centrifuged. ApoE released from the cell surface (expressed in ng/mg cell protein) was determined by ELISA. Results are expressed as mean±SEM of two to four determinations per donor.

Cholesterol accumulation in macrophages. In the unloaded state, macrophages from all three homozygous phenotypes contained ~ 200 μg free cholesterol per milligram cell protein and very little cholesteryl ester. No significant differences existed between the cholesterol concentrations of the unloaded cells of the three phenotypes (data not shown). On loading with 80 μg/ml AcLDL for 48 h, the amount of free cholesterol

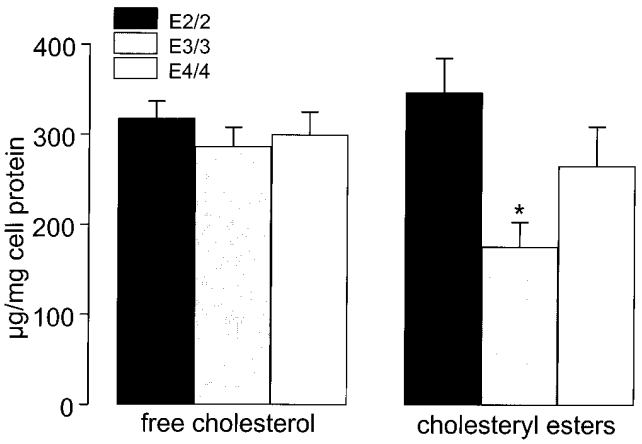

Figure 4. Free and esterified cholesterol content in cholesterol-loaded human monocyte–derived macrophages. Cells of five donors per phenotype were incubated in serum-free RPMI 1640 containing 80 μg/ml AcLDL for 48 h, washed and incubated in serum-free RPMI 1640 for a further 24 h. Cholesterol and cholesteryl ester levels were determined by HPLC. Results are expressed as mean±SEM of three to seven determinations per donor. All values were significantly higher in AcLDL-loaded cells compared with unloaded cells ($P < 0.05$ for free cholesterol, $P < 0.001$ for cholesteryl ester). The differences among the three phenotypes in cholesteryl ester were statistically significant (ANOVA, $P < 0.01$). *$P < 0.01$ versus E2/2; $P < 0.05$ versus E4/4.

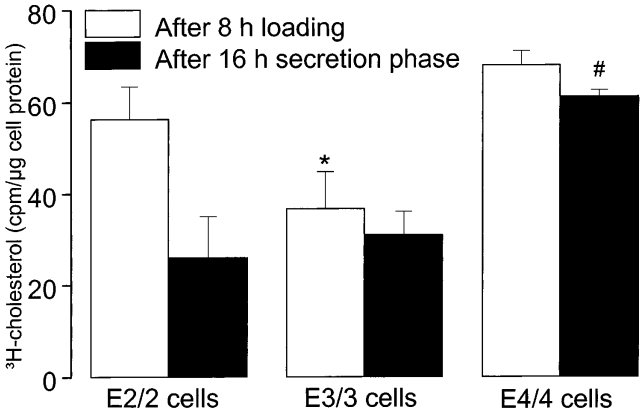

Figure 5. Uptake and secretion of total (free plus esterified) ^{3}H-cholesterol in cholesterol-loaded human monocyte–derived macrophages. Cells of four donors per phenotype were incubated with 200 μg/ml ^{3}H-AcLDL for 8 h (*loading*), washed, and incubated in serum-free RPMI 1640 overnight (*secretion phase*). At the end of each incubation, cells were harvested, delipidated in chloroform/methanol, 1:2, dried down, and redissolved in chloroform. Free and esterified ^{3}H-cholesterol were separated by TLC and quantified by scintillation counting. Results are expressed as mean±SEM of two to three measurements per donor. *$P < 0.01$ versus E2/2; $P < 0.05$ versus E4/4; #$P < 0.05$ versus E2/2 and E3/3.

and cholesteryl ester increased significantly in all three phenotypes ($P < 0.05$ for free cholesterol, $P < 0.001$ for cholesteryl ester; Fig. 4). The increase in free cholesterol in the E2/2 cells (43%) was significantly greater than that in either the E3/3 or E4/4 cells (29% in each case, $P < 0.05$ for comparison between phenotypes). By contrast, cholesteryl ester increased to a lesser extent in the E3/3 cells (after loading 176±27 μg/mg cell protein) than in E2/2 or E4/4 cells (after loading 347±38 and 266±43 μg/mg cell protein, respectively; $P < 0.01$ for comparison between phenotypes, Fig. 4).

Incubation of cells with ^{3}H-cholesterol–AcLDL. To assess whether phenotype-specific differences exist in the ability of human macrophages to accumulate and excrete exogenous cholesterol, the cells were incubated for 8 h with 200 μg/ml ^{3}H-cholesterol AcLDL and then for a further 16 h in lipoprotein- and serum-free medium. After loading, E3/3 cells accumulated about 40% less total ^{3}H-cholesterol than did cells of the other two phenotypes, both of which accumulated approximately the same amount (Fig. 5). However, during a further 16 h incubation in serum- and lipid-free medium, E2/2 cells secreted 52% of their accumulated cholesterol into the medium, compared with 14% in E3/3 and 10% in E4/4 cells ($P < 0.01$ in each case). Moreover, E4/4 cells retained about twice as much ^{3}H-cholesterol (61.3±1.3 cpm/μg protein) as did E2/2 or E3/3 cells (26.1±8.9 and 31.1±5.0 cpm/μg protein, respectively; $P < 0.05$ in each case). When ^{3}H-cholesterol excretion over the 16 h incubation period was analyzed in terms of free ^{3}H-cholesterol and ^{3}H-cholesteryl ester, it was found that E2/2 and E3/3 cells lost both free ^{3}H-cholesterol (−62 and −17%, respectively) and cholesteryl ester (−35 and −4%, respectively; Fig. 6). E4/4 cells also lost free ^{3}H-cholesterol (−22%), but actually continued to accumulate ^{3}H-cholesteryl ester during this period (+15%). Thus the loss of free ^{3}H-cholesterol was in the order E2/2 > E3/3 ≅ E4/4, while the loss of ^{3}H-cholesteryl ester was in the order E2/2 > E3/3 > E4/4. Not surprisingly ∼ 20% more radioactivity accumulated in the culture medium of E2/2 than in that of E3/3 or E4/4 cells (Fig. 6, *inset*).

Uptake of ^{3}H-cholesterol from macrophage-conditioned media. It is possible that the lipoprotein particles secreted by the different phenotypes differ in their ability to bind to cell surface receptors and thus in their aptitude for reuptake by the cells. For this reason, we incubated unloaded E3/3 cells for 24 h in the presence of media which had been conditioned by incubation for 16 h with preloaded cells of each phenotype. Preloading was performed by incubation with ^{3}H-cholesterol–labeled AcLDL, as described above. Uptake of ^{3}H-cholesterol from macrophage-conditioned media by E3/3 cells was of the order E4/4 medium > E3/3 medium > E2/2 medium (Fig. 7).

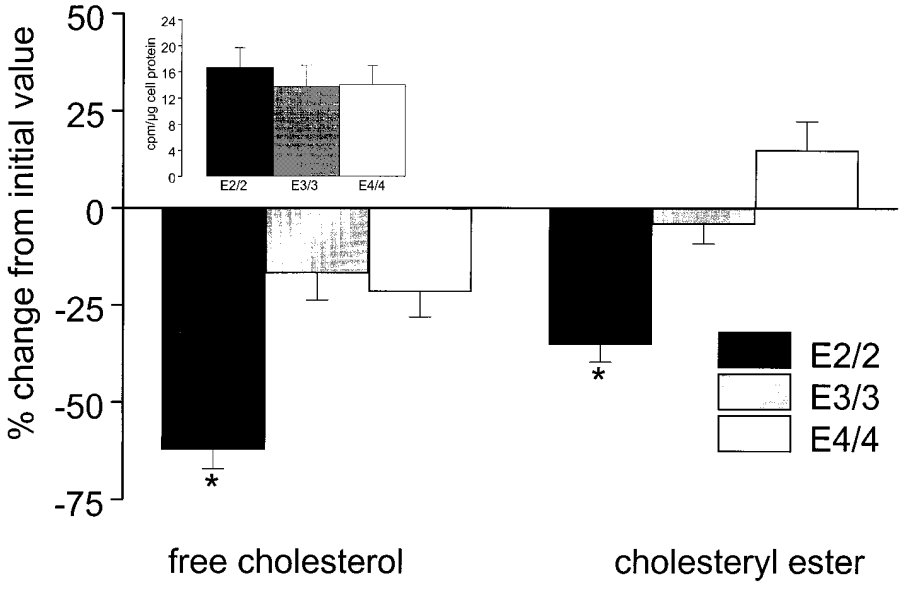

Figure 6. Relative changes in ^{3}H-free cholesterol and cholesteryl ester content in cholesterol-loaded human monocyte-derived macrophages after a secretion phase. Cells were incubated and treated as described in Fig. 5. Percentage changes were measured by comparing the values at the start and end of the secretion phase. The inset shows the accumulation of total (free plus esterified) ^{3}H-cholesterol in the secretion medium. Results are expressed as mean±SEM of two to three measurements per donor. *$P < 0.01$ versus E3/3 or E4/4.

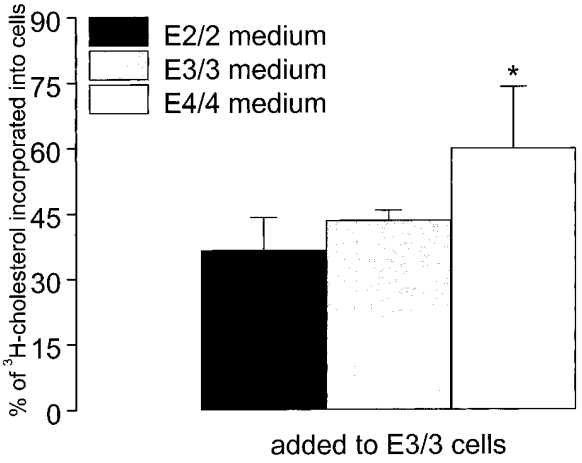

Figure 7. Percentage uptake of total (free plus esterified) ^{3}H-cholesterol into unlabeled E3/3 cells incubated with conditioned media from each phenotype. Cells were incubated and treated as described in Fig. 5. Fresh E3/3 cells were then incubated for a further 24 h with the conditioned media of each phenotype harvested after the secretion incubation. All values were normalized for cellular protein content. Results are expressed as mean±SEM of two to three measurements per donor. $*P < 0.05$ versus E2/2 and E3/3.

Discussion

This study found that the metabolism of cholesterol and apoE differs markedly among macrophages of the three homozygous apoE phenotypes. In unloaded cells, the net secretion of apoE into the medium of E3/3 cells was greater than that of E2/2 cells (Fig. 2), despite similar apoE mRNA levels in all three cell types (Fig. 1). Since translational control of specific mRNAs is uncommon in eukaryotes (33), it is likely that in unloaded E2/2 cells, a portion of the apoE is destined not for secretion but for intracellular degradation. This conclusion is also supported by the results of pulse-chase experiments with ^{35}S-methionine (Table II).

Lysosomal degradation of apoE is known to occur in macrophages (31, 32). Lucas and Mazzone have also shown (34) that apoE binds to the proteoglycan matrix of macrophages and that this may control net secretion of apoE since this trapped apoE is susceptible to rapid cellular degradation. As shown by our pulse-chase experiments, newly synthesized apoE3 and apoE4 disappear from the cells (and appear in the medium) more rapidly than apoE2 (Fig. 3). ApoE2, however, accumulates in the medium whereas apoE3 and apoE4 peak at 2 h and then diminish indicating either reuptake or degradation or both. In addition, more apoE4 than apoE3 or apoE2 was releasable by heparinase from the cell surface at 4°C (Table III). This increased cell surface binding provides evidence for enhanced reuptake of apoE4 from the medium and may also explain the slightly lower levels of apoE4 found in the media of unloaded cells (Figs. 2 and 3).

In the unloaded state, the differences in apoE secretion have no impact on cholesterol homeostasis since all three phenotypes contained similar amounts of free cholesterol and virtually no cholesteryl ester. The situation becomes very different upon loading of macrophages with AcLDL. In the loaded state, the level of apoE mRNA increased 4.3-fold in the E4/4 and 2.2-fold in the E3/3 cells, but not in the E2/2 cells (Fig. 1). In E3/3 and E4/4 cells, the increase in apoE mRNA was associated with increased secretion of apoE into the culture medium (Figs. 1 and 2). In the loaded E2/2 cells, apoE accumulation in the medium increased twofold despite a constant level of apoE mRNA, probably because of redirection of apoE from the degradatory to the secretory pathway.

The increase in apoE mRNA and protein secretion in response to cholesterol loading of human macrophages is in agreement with the work of Mazzone et al. (35, 36), who showed increased synthesis of apoE on cholesterol loading in mouse peritoneal macrophages. However, our results are in sharp contrast to a report by Zhang et al. (37), who failed to find an increase in apoE mRNA levels and protein secretion on cholesterol loading in human monocyte–derived macrophages cultured under conditions similar to our own. Under our experimental conditions, cells secreted between 0.4 and 2.0 μg apoE/mg cell protein per 24 h. This agrees with previous values from the literature (38), whereas the values reported by Zhang et al. are some three to five times greater. Endotoxin is known to inhibit apoE secretion, and Zhang et al. speculate that their use of culture serum selected for low levels of endotoxin may have led to maximal expression of apoE in their unloaded cells, which was not further increased on cholesterol loading. However, the medium used in our experiments was also endotoxin-free. Moreover, the same medium and pooled human serum was used throughout. Thus, we are unable to explain the discrepancy between these findings and our own.

The phenotype-related differences in apoE mRNA transcription and protein secretion after macrophage loading are paralleled by differences in cholesterol and cholesteryl ester accumulation (Fig. 4). Free cholesterol increased significantly in all cell types, but to a greater extent in E2/2 than in E3/3 or E4/4 cells. Cholesteryl ester increased dramatically on AcLDL loading in all three cell types, but in E2/2 > E4/4 > E3/3. Our findings in this respect agree with those of Zhang et al. who also demonstrated cholesterol efflux from human macrophages in the absence of exogenous acceptors (37). Spontaneous cholesterol efflux from loaded cells in the absence of extracellular acceptors was in the order E2/2 > > E3/3 ≅ E4/4. These phenotype-related differences in apoE synthesis and secretion as well as in cholesterol accumulation raise the question as to whether the differences in apoE production caused the differences in uptake and secretion of cholesterol or vice versa. ApoE gene expression and apoE synthesis are thought to be regulated by intracellular free cholesterol (39). However, on cholesterol loading, E2/2 cells did not increase the level of apoE mRNA despite their pronounced accumulation of cholesterol. This finding would appear to exclude a regulatory role for intracellular free cholesterol in the regulation of *apoE* gene transcription. However, it is possible that in human macrophages *apoE* transcription is governed by an active pool of intracellular cholesterol, or a metabolite thereof, which is not quantified when the total free cholesterol content is measured. Moreover, apoE2/2 cells exported cholesterol most effectively (Fig. 6). Therefore, the failure of apoE2/2 cells to increase *apoE* gene expression on cholesterol loading may be related to the fact that the active pool of cell cholesterol, or a metabolite thereof, regulating apoE may not increase to such an extent as to trigger enhanced *apoE* gene expression. On the other hand, the apoE mRNA level and protein secretion increased more in

E4/4 cells than in the other two phenotypes. This may be a compensatory mechanism, because E4/4 cells showed the least effective cholesterol efflux, perhaps because of a greater reuptake of apoE4-containing cholesterol-rich particles (Fig. 7, Table III).

Although the reasons for the phenotype-specific differences in *apoE* gene transcription and apoE metabolism are unknown, it is clear from our experiments that these quantitative differences, together with qualitative differences in apoE structure, affect the cholesterol homeostasis of cells. Both the quantification of cellular cholesteryl ester after incubation with AcLDL and the ^{3}H-cholesterol–AcLDL experiments indicate that E2/2 cells accumulate more cholesteryl ester than cells of the other two phenotypes, possibly because of a slower basal apoE secretion rate. However, they dispose of it more efficiently by means of a later-onset mechanism of cholesterol efflux. ApoE2 was found to accumulate in medium, unlike the other two isoforms. One explanation for this increased efficiency of E2/2 cells in exporting cholesterol is the resistance of apoE2 to reuptake because of its well-known low affinity for apoE receptors. The data from our crossover experiment agreed with this model (Fig. 7). E3/3 cells accumulated the least cholesterol, probably because of their relatively high basal level of apoE secretion. Although cholesterol loading of E4/4 cells resulted in the highest apoE secretion, cholesterol efflux was least from these cells. One reason for this may be an increased reuptake of secreted cholesterol, possibly in conjunction with the apoE4 protein secreted by these cells. This increased uptake may be partly related to the increased binding of apoE4 to the cell surface, probably in complexes with heparan sulfate proteoglycans, as revealed by studies using heparinase and suramin (Table III).

Efflux of cholesterol from cells in vivo is a complex process and is greatly influenced by the presence of cholesterol acceptors (40, 41). It has been argued that macrophage-derived apoE facilitates cholesterol efflux only in the presence of HDL. Our data, together with those of Kruth and colleagues (18, 37), clearly indicate that, at least in human monocyte–derived macrophages, cholesterol efflux is facilitated by endogenous apoE even in the absence of additional cholesterol acceptors. Nevertheless, it must be recognized that in vivo, macrophages in the arterial wall are surrounded by several classes of lipoproteins, including HDL. Such acceptors may also affect intracellular cholesterol transport and, thus, apoE production. HDL, for example, promotes the active transport of cholesterol from intracellular pools to the cell membrane (42) and diverts apoE from the degradatory to the secretory pathway (43). Thus, in the presence of exogenous acceptors, many of the features we have observed may be modified.

In conclusion, our data demonstrate significant differences in the expression of the *apoE* gene, in apoE secretion and in the storage and secretion of cholesterol by human monocyte–derived macrophages. In view of the association between apoE4 and CHD (4), we hypothesize that the less effective cholesteryl efflux of E4/4 macrophages (Figs. 5 and 6) may facilitate the development of atherosclerosis. Previous work has demonstrated the atheroprotective role of macrophage apoE (13, 14), particularly that produced locally within the arterial wall (15, 16). The risk for Alzheimer's disease is increased in individuals carrying one or two ϵ4 alleles. If the differences found here for macrophages also apply to astrocytes in the central nervous system, which are known to produce apoE (44), then it is possible that increased production of apoE by astrocytes might contribute to the pathogenesis of Alzheimer's disease in persons carrying an ϵ4 allele. E2/2 macrophages accumulated the largest amounts of cholesterol during loading with AcLDL, but were also most efficient in cholesterol efflux. This efficient removal of cholesterol, together with the lower LDL cholesterol levels seen in ϵ2 carriers may help explain the reduced cardiovascular risk associated with this allele. In addition, the reduced secretion of apoE in E2/2 individuals may in some way be related to the reduced incidence of Alzheimer's disease in subjects carrying an ϵ2 allele (45).

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