Synthesis and secretion of apoE in thioglycolate-elicited mouse peritoneal macrophages: effect of cholesterol efflux

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Abstract ApoE synthesis and secretion, as a function of cellular cholesterol content and cholesterol efflux, was studied in thioglycolate-elicited mouse peritoneal macrophages. As expected, loading elicited macrophages with cholesterol induced a 5-fold increase in apoE secretion and a 2.5-fold increase in cellular apoE content over a 5-h period. Treatment of cholesterol-loaded cells with HDL_3 further increased apoE secretion 1.7-fold and decreased cellular cholesterol by 20%. Treatment of cholesterol-loaded cells with HDL_3 and SAH 58.035 (an ACAT inhibitor) increased apoE secretion 2.4-fold and decreased cellular cholesterol content by 35%. Treatment of the cells with the ACAT inhibitor alone suppressed apoE secretion by 40% but did not change cellular cholesterol content. Northern blot analysis of RNA indicated that cholesterol loading increased apoE mRNA 2-fold. ApoE mRNA levels were not further affected by treatment with HDL_3 and/or the ACAT inhibitor. Cholesterol-loaded cells, in the absence of HDL_3, secreted apoE into the media in two fractions as determined by column chromatography: a large molecular weight complex, (larger than HDL), and an essentially lipid-free protein. In the presence of HDL_3, the cells secreted apoE in three fractions: a large molecular weight complex, an essentially lipid-free protein, and over 50% of apoE associated with HDL. In the process, HDL_3 became larger and eluted in a position identical to that of HDL_2. A small amount of HDL_3-derived material was also transformed to an LDL-size particle. Incubation of HDL_3 in the absence of cholesterol-loaded cells did not produce these changes. It is concluded that cholesterol-loading increases apoE mRNA content and apoE synthesis. ApoE synthesis and secretion, but not apoE mRNA levels are further increased by HDL-mediated cholesterol efflux. —**Dory, L.** Synthesis and secretion of apoE in thioglycolate-elicited mouse peritoneal macrophages: effect of cholesterol efflux. *J. Lipid Res.* 1989. **30:** 809–816.

Supplementary key words macrophages • apoE mRNA • ACAT • HDL

Apolipoprotein (apo) E is a major apolipoprotein component of circulating lipoproteins (1). A key function of apoE is to mediate the receptor-specific uptake of cholesterol-rich lipoproteins from the circulation (2–4). ApoE also appears to play an important role in cholesterol transport within peripheral tissues and reverse transport of cholesterol from the periphery to the liver. Evidence for this comes from studies demonstrating apoE synthesis and secretion by macrophages, rat brain astrocytes, and cells involved in nerve injury repair of the peripheral and central nervous system (5, 6). Peripheral tissues may contribute a significant amount to the body pool of apoE; the capacity of these tissues to synthesize apoE, as demonstrated by the presence of apoE mRNA, has been shown in a number of laboratories (7–9). In vitro evidence for apoE synthesis by various human tissue slices has also been reported (10). Direct, in vivo evidence for synthesis of apoE by peripheral tissues has been demonstrated in dogs (11). It is not clear whether the role of apoE is essentially restricted to targeting the cholesterol associated with it, or includes a function as a cholesterol transporter and promoter of cholesterol efflux from peripheral cells. It seems, however, that it is not essential for the latter two, as apoE-free HDL is an efficient acceptor and carrier of effluent cholesterol (12).

Cultured macrophages provide a particularly well-suited model for the study of the involvement of apoE in cholesterol transport. Macrophage-derived, cholesterol-loaded foam cells are a prominent feature of the arterial fatty streak and likely play an important role in the subsequent development of the fibrous plaque (13). ApoE synthesis by macrophages appears to be regulated by a number of factors, including: *1*) cellular cholesterol content (5), *2*) immunological stimulation (14), *3*) cell dif-

Abbreviations: apo, apolipoprotein; ACAT, acyl coenzyme A: cholesterol acyltransferase; DMEM, Dulbecco's Modified Eagle's Medium; FBS, fetal bovine serum; PBS, phosphate-buffered saline; TCA, trichloroacetic acid; DMSO, dimethyl sulfoxide; acLDL, acetylated low density lipoproteins; HDL, high density lipoproteins; HDL_c, cholesterol-induced HDL; d, density; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; LCAT, lecithin: cholesterol acyltransferase; TG, thioglycolate.

ferentiation (15), and demands of tissue repair (6). In the present study, the regulation of apoE synthesis and secretion by cholesterol efflux was examined in thioglycolate-elicited mouse peritoneal macrophages. In addition, the nature of the newly secreted apoE was investigated by column chromatography.

MATERIALS AND METHODS

L-[^{35}S]Methionine (sp act > 1100 Ci/mmol), and [α-^{32}P]deoxycytidine-5'-triphosphate (sp act ~ 3000Ci/mmol) were obtained from NEN Research Products and ICN Biomedicals, Inc., respectively. Culture media, including Dulbecco's phosphate-buffered saline, Dulbecco's minimal essential media, methionine-free essential media, and fetal bovine serum were obtained from Gibco. Enzymes for cholesterol determination (cholesterol esterase and oxidase) were obtained from Boehringer Mannheim Biochemicals. The ACAT inhibitor SAH-58.035 was provided by Dr. John Heider from the Sandoz Research Institute. Protein A-6% Agarose complex was purchased from Repligen. Column chromatography was carried out in 10% agarose (A-0.5), purchased from Bio-Rad.

Cell culture

Mouse peritoneal macrophages were collected from male Swiss-Webster mice (18–20 g), obtained from Harlan, 4 days after an intraperitoneal injection of 1.5 ml of sterile 4% thioglycolate broth. All subsequent procedures were carried out under sterile conditions. The cells were collected by intraperitoneal lavage with sterile DPBS. The lavage fluid from several mice was pooled and centrifuged for 10 min at 800 *g*. The pelleted cells were resuspended in DMEM supplemented with 20% FBS, counted, and plated in 35-mm dishes at 5–10 × 10^6 cells/dish. Nonadherent cells were removed after 3 h by several washes with DMEM, and the macrophages were incubated overnight in the original plating media (DMEM- 20% FBS).

The next day the experimental cells were incubated in the presence of acetylated human LDL (at 100 μg/ml) in DMEM. Control cells were incubated in DMEM alone. After a 24-h loading period, the cells were incubated in the test media for 5 h. The test media consisted of methionine-free DMEM containing 20 μCi of [^{35}S]-methionine/ml (2ml/dish) and, depending on the desired conditions, the ACAT inhibitor SAH-58.035 (5 μg/ml) and /or human plasma HDL_3 (d 1.125–1.21 g/ml), at 100 μg/ml. The ACAT inhibitor was dissolved in DMSO; the final concentration of DMSO in the media was 0.05%. Both human acLDL and HDL_3 were extensively dialyzed against DMEM prior to their addition to the incubation media. All incubations were carried out at 37°C in a humidified atmosphere containing 5% CO_2.

Cellular cholesterol and protein determinations

At the end of the incubations the cells were washed three times with ice-cold PBS, scraped off with a rubber policeman, and resuspended in PBS (final volume, 2 ml). An aliquot of this suspension was used directly for cell protein mass determination (16), while another aliquot was used for lipid extraction (17). The dried lipid residue was re-extracted with chloroform, dried, and dissolved in isopropanol. Free and total cholesterol mass were determined by fluorometric enzymatic assay (18), and the cholesteryl ester mass was calculated.

Total protein and apoE synthesis and secretion

Total media and cellular protein synthesis were determined by TCA precipitation of an aliquot of media or cell suspension. To facilitate recoveries, 0.5% BSA solution was added to each aliquot prior to the addition of 20% TCA. The precipitates were extensively washed in 10% TCA, dissolved in 0.5 M phosphate buffer and an aliquot was taken for scintillation counting. Alternatively, the TCA precipitates were dissolved in the SDS-PAGE protein solvent (19), neutralized with 1 M NaOH (until bromophenol blue turned blue), and boiled for 5 min; the proteins were resolved by SDS-PAGE (19). Total media apoE was immunoprecipitated in the presence of 0.05% Triton X-100, 1 μM PMSF, and 0.2 U of aprotinin at 4°C for 24 h, followed by addition of excess protein A–agarose to remove the immune complex. The agarose–protein A–immune complex was washed twice with a 0.05 M Tris-HCl buffer, pH 7.4, containing 0.15 M NaCl, 5 mM EDTA, 1 mM PMSF, and 0.05% Triton X-100. The last wash was carried out in the absence of Triton X-100. The Laemmli protein solvent was added directly to the washed beads, the mixture was boiled for 5 min and centrifuged in a microfuge, and the protein mixture was resolved by SDS-PAGE. Slab gels processed for fluorography were first soaked in En^{3}Hance and dried. ApoE was quantitated by scanning the X-ray film, using the LKB 2202 Ultroscan Laser densitometer and a Hewlett-Packard integrator, and the results were expressed as % of secretion (or synthesis) by control cells. A typical appearance of a fluorograph of total media proteins (TCA precipitates) and apoE immunoprecipitates in normal and cholesterol-loaded macrophages is shown in **Fig. 1**.

Cellular apoE was immunoprecipitated in a similar fashion, except that 1% Triton X-100 was used and the cell nuclei were pelleted prior to the immunoprecipitation. In one set of experiments the cell lysates as well as the media were pre-cleared by addition of nonimmune goat serum, followed by protein A-agarose, to minimize

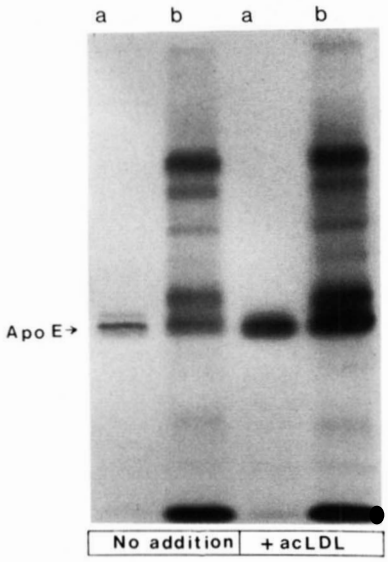


Fig. 1. Fluorograph of an SDS-PAGE slab gel (11%) of media immunoprecipitable apoE (a) or TCA-precipitable total protein (b) secreted by thioglycolate-elicited control (No addition) and cholesterol-loaded (+ acLDL) mouse macrophages. Equal aliquots (0.1 ml) of the media were used for all precipitation procedures. Cells were cultured as described in Materials and Methods.

background. This however did not result in a significant improvement and the procedure was not continued. The efficiency of immunoproecipitation in the media and cell extracts was also checked by addition of trace amounts of ^{125}I-labeled rat apoE. In each case over 90% of the added apoE was immunoprecipitated.

In some experiments the macrophage-conditioned test media from six dishes were concentrated (approximately 10-fold) by centrifugal ultrafiltration (using 10K Centri-Cell filters; Polysciences, Inc.) and an aliquot (1.1 ml) was immediately applied to a 0.9 × 100 cm column of 10% agarose. Column fractions were monitored for total [^{35}S]-methionine incorporation (by direct counting of small aliquots) as well as for apoE, by immunoprecipitation, followed by scintillation counting. Pooled column fractions were also dialyzed, lyophilized, solubilized under reducing conditions, and analyzed by SDS-PAGE followed by fluorography.

Northern blot analysis of cellular RNA

Total cellular RNA was extracted from individual dishes by the acid phenol–guanidinium isothiocyanate method (20). One and 2 μg of total RNA from each experimental group (from three separate experiments) were separated by agarose electrophoresis, using formaldehyde-containing 1.2% agarose. Northern blotting was carried out overnight, using nitrocellulose (PH75, Schleicher & Schuell). After baking at 80°C for 2 h, the sheets were prehybridized overnight and hybridized with nick-translated mouse apoE cDNA (nearly full-length probe 2C1-apoE) (21) for 48 h at 42°C (22). The washed nitrocellulose sheets were exposed to X-Omat AR film (Kodak) for various times. Sheets were also re-probed with chicken beta-actin (23). The X-ray films were densitometrically scanned; each blot was scanned at four arbitrary positions and the values obtained were averaged.

RESULTS

ACAT inhibition and cellular cholesterol distribution

Compound SAH-58.035 was an effective inhibitor of ACAT in thioglycolate-elicited mouse peritoneal macrophages. As shown in **Fig. 2**, increasing time of exposure of cholesterol-loaded cells to the inhibitor (at 5 μg/ml; 2 ml/dish) resulted in a progressive redistribution of cellular cholesterol mass in favor of unesterified cholesterol. Total cholesterol mass remained virtually unchanged. Extended exposure to the inhibitor (over 36 h, not shown) led to significant morphological changes (at light microscopic level) and cell death (as estimated by cell detachment from the bottom of the dish). This effect did not occur in the presence of exogenous cholesterol acceptor in the media.

ApoE synthesis

A convenient period of [^{35}S]methionine incorporation into immunoprecipitable apoE was determined in pre-

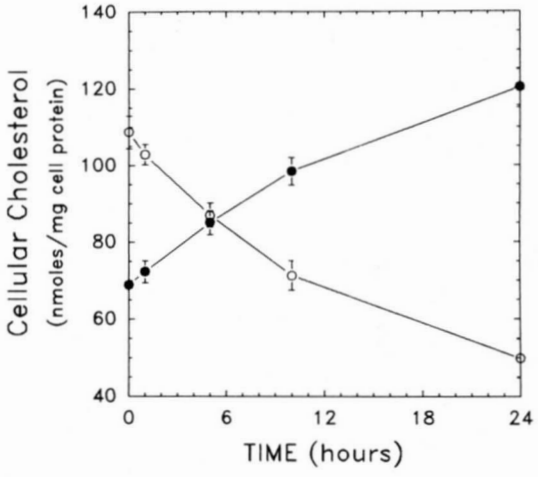


Fig. 2. The effect of the ACAT inhibitor SAH-58.035 on cellular cholesterol distribution. Thioglycolate-elicited macrophges were loaded with human acLDL (100 μg/ml) for 24 h, washed, and incubated for the indicated periods of time with DMEM containing SAH-58.035 at 5 μg/ml (2 ml/dish). At each time point the cells were washed with ice-cold saline, scraped off, and resuspended in 2 ml of saline. Cellular free (●) and total cholesterol were determined as described in Materials and Methods. Cholesteryl ester content (○) was calculated. The values represent means ± SEM for four dishes in each group.

liminary experiments. Results of a typical experiment are shown in **Fig. 3.** As the fluorograph indicates, incorporation of [^{35}S]methionine into secreted apoE by thioglycolate-elicited macrophages was virtually linear for 10 h. Subsequently, in the experiments reported below, a 5-h test period was used. This time period also allowed for a significant redistribution of cholesterol mass in the presence of the SAH-58.035, as shown above.

Secretion of apoE into the media was examined in thioglycolate-elicited, cholesterol-loaded cells in which free cholesterol efflux was modulated by incubating the cells in the presence of HDL_3 alone or in combination with the ACAT inhibitor. The effect of SAH-58.035 alone was also determined. The results were compared to cells not loaded with cholesterol. In addition, TG-elicited cells were compared to resident macrophages. As shown in **Fig. 4**, thioglycolate-elicited macrophages secreted approximately 10 times as much apoE as resident macrophages. Cholesterol-loading of TG-elicited macrophages induced a further 5-fold increase in apoE secretion. Presence of HDL_3 in the incubation media induced an additional 2- and 2.5-fold increase in apoE secretion (relative to cholesterol-loaded cells), in the absence or presence of SAH 58.035, respectively. SAH 58.035 treatment alone of cholesterol-loaded cells inhibited apoE secretion somewhat.

These observations suggested that the extent of cholesterol efflux may play an important role in the degree of apoE secretion. In **Fig. 5** cellular cholesterol content and mass distribution (A) are related to cellular apoE content and apoE secretion (B). The results represent an average of four experiments. As shown, cholesterol loading increased the total cholesterol content of the cells 3-fold (see panel A), much of it in the form of cholesteryl ester. ApoE secretion and cellular apoE content in these cells was over 5- and 2-fold higher, respectively, than the values found in control cells (panel B). Treatment of cholesterol-loaded cells with HDL_3 resulted in a 20% decrease in cell

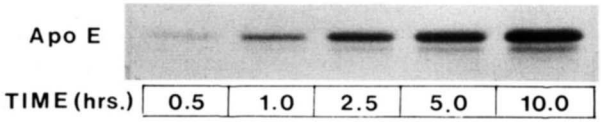


Fig. 3. Secretion of [^{35}S]methionine-apoE by thioglycolate-elicited mouse macrophages as a function of time. Macrophages were obtained as described in Materials and Methods. Following the overnight incubation in DMEM/20% FBS, the cells were washed 3 × with DMEM and incubated for 24 h in the absence of acLDL. On day 3 the cells were incubated in the test media consisting of methionine-free DMEM containing 40 μCi [^{35}S]methionine/dish for the indicated periods of time. At the end of each time period, aliquots (2 × 0.1 ml) were taken for apoE immunoprecipitation. The immunoprecipitates were resolved by SDS-PAGE and the gel was fluorographed. Total protein secretion was determined by TCA precipitation and remained linear over the 10-h period investigated (not shown).

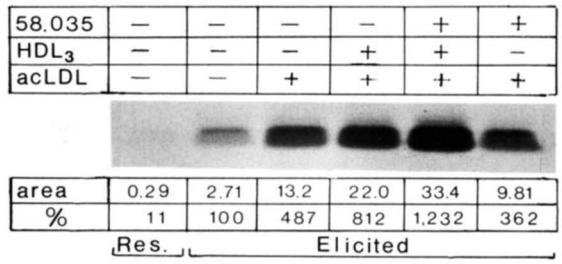


Fig. 4. [^{35}S]Methionine-labeled apoE secretion by resident and thioglycolate-elicited mouse macrophages under the indicated experimental conditions. Resident and TG-elicited cells were compared in the absence of cholesterol loading (– acLDL). These were compared to cholesterol-loaded (+ acLDL, over 24 h) TG-elicited cells incubated in the presence (+) or absence (–) of HDL_3 (100 μg/ml) and/or SAH-58.035. Cells were loaded with cholesterol (acLDL) on day 2 and HDL_3 and/or 58.035 was added in the test media for 5 h on day 3. The test media also contained 40 μCi of [^{35}S]methionine/dish. The fluorographs of the apoE immunoprecipitates, resolved by SDS-PAGE, were scanned. The areas under each curve were evaluated (see Materials and Methods) and are shown in the bottom of the figure. The results were also expressed relative to apoE secretion by elicited cells in the absence of cholesterol loading (%).

cholesterol content, and stimulated an additional 1.7-fold increase in apoE secretion, and a 50% decrease in cellular apoE content. Treatment of the cells with both HDL_3 and SAH 58.035 resulted in a loss of 35% of their cholesterol content through efflux and a 2.4-fold increase in apoE secretion. Exposure of cholesterol-loaded cells to SAH-58.035 alone had virtually no effect on cellular cholesterol content (although it had a profound effect on cholesterol mass distribution). In the absence of cholesterol efflux from these cells, apoE secretion was significantly decreased, when compared to any other cholesterol-loaded cell group. It remained over 3-fold increased, however, when compared to the control cells.

ApoE mRNA

In order to determine whether changes observed in the rates of apoE synthesis and secretion are mediated at the transcriptional level, the apoE mRNA content of the cells in the various experimental groups was estimated by Northern blot analysis of total cellular RNA. A typical Northern blot analysis as well as the cumulative results of three separate experiments are shown in **Fig. 6**. Cholesterol loading induced a twofold increase in the cellular apoE mRNA content; further exposure to HDL_3 and/or the ACAT inhibitor did not change apoE mRNA levels, despite the significant changes in apoE synthesis and secretion shown above.

ApoE distribution in the media

The nature of the newly synthesized apoE secreted by the cells into the media was investigated by column chro-

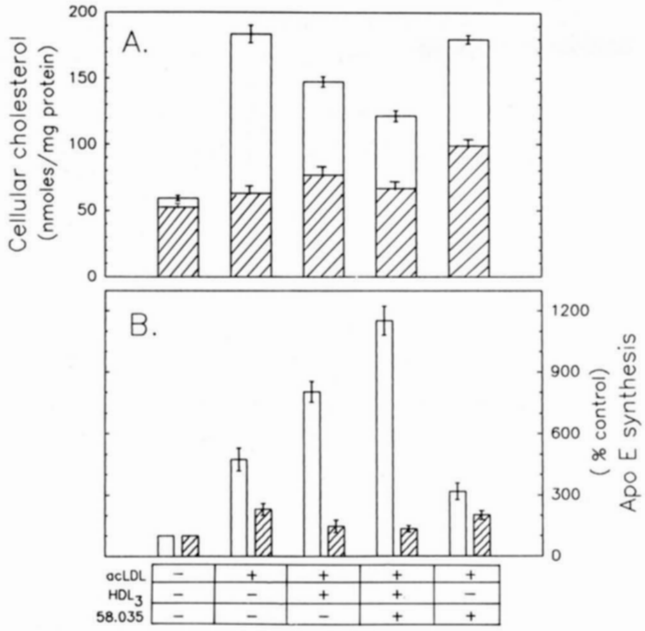
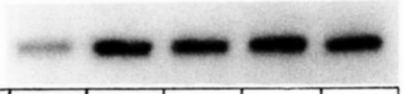


Fig. 5. Cellular cholesterol mass distribution (A) and apoE synthesis (B) in thioglycolate-elicited macrophages under the indicated experimental conditions. For panel A, cellular free cholesterol (▨) and cholesteryl esters (□) were determined as described in Materials and Methods. Panel B: the relative amounts of immunoprecipitable media (□) and cellular (▨) apoE under these conditions are shown. ApoE immunoprecipitations were carried out as described in Materials and Methods. Each bar represents an average of four separate experiments with duplicate determinations in each. Error bars indicate SEM.

matography in 10% agarose. Prior to the chromatography, the media were concentrated (ca. 10-fold) by low-speed centrifugal ultrafiltration.

Results of a series of typical experiments are shown in **Fig. 7.** The column was calibrated prior to the experiments and the elution positions of human LDL, HDL_3, albumin, and rat delipidated apoE monomer are indicated in panel A. Cholesterol-loaded cells incubated in



ac LDL	–	+	+	+	+
HDL_3	–	–	+	–	+
58.035	–	–	–	+	+
Area (%)	100	219±13	206±8	220±13	196±9

Fig. 6. Northern blot assay of total RNA harvested from cells cultured as described in the legend for Fig. 4, except without [^{35}S]methionine. Three separate experiments were carried out and each time 1 and 2 μg of RNA were resolved by 1.2% agarose electrophoresis in the presence of formaldehyde. The prehybridized nitrocellulose paper was hybridized with nick-translated mouse apoE cDNA, as described in Materials and Methods. A typical analysis (2 μg RNA/lane) is shown in the upper part of the figure. For each run several exposures were taken and each lane was scanned at four separate positions to obtain the final values. The areas under each blot were normalized to those obtained from control cells. The relative amount of beta actin mRNA in each sample was the same (not shown).

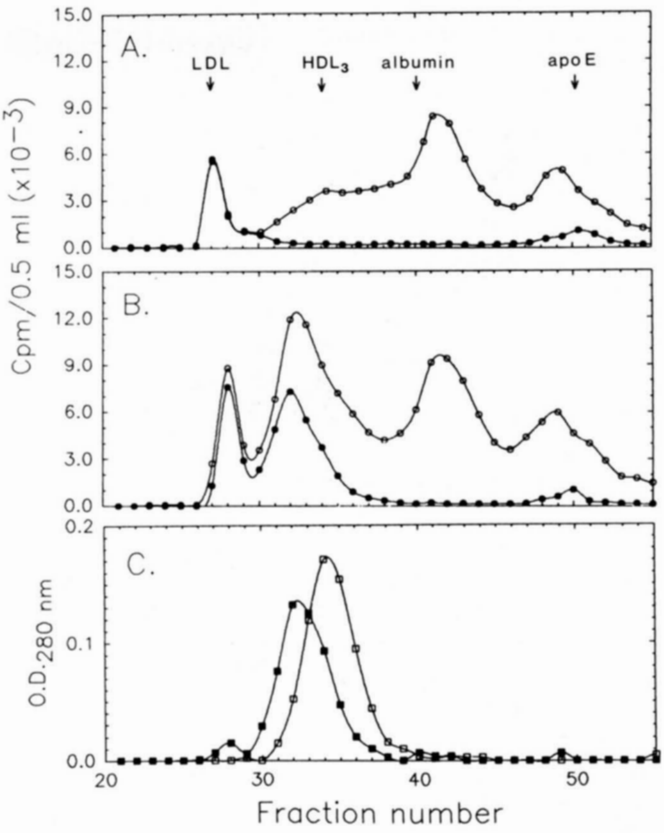


Fig. 7. Column chromatography of concentrated (10 ×), macrophage-conditioned test media, isolated after a 5-h incubation period. Macrophage-conditioned test media without HDL_3 (A) or with HDL_3(B) were analyzed using a 0.9 × 100 cm column of 10% agarose, collecting 1.1-ml aliquots. The elution positions of various isolated proteins and lipoproteins are indicated by arrows in panel A. Total ^{35}S radioactivity (○) was assayed directly by scintillation counting. ^{35}S-Labeled apoE radioactivity (●) was determined by scintillation counting of immunoprecipitated apoE in each fraction. The elution of HDL_3 incubated under identical conditions but in the absence of cells is shown in panel C (□) and is compared to that of HDL_3 incubated in the presence of cholesterol-loaded cells (■).

the absence of a cholesterol acceptor in the media secreted apoE as a large molecular weight complex, eluting in the void volume of the column, and a much smaller fraction, corresponding to lipid-free apoE monomer (panel A). The presence of HDL_3 in the test media (panel B) led to a significant re-distribution of secreted apoE. Although a substantial proportion (35–40%) was still secreted as a large molecular weight complex and a small amount continued to be secreted in an apparently lipid-free form, over 50% of the media apoE eluted in association with the HDL particles added to the incubation media. The acquisition of apoE by HDL was accompanied by a significant change in the size of HDL (see panel C). Exposure of human HDL_3 to cholesterol-loaded macrophages transformed it consistently to a larger sized particle, corresponding to HDL_2. A small amount of material eluting in the void volume was also consistently obtained. HDL_3

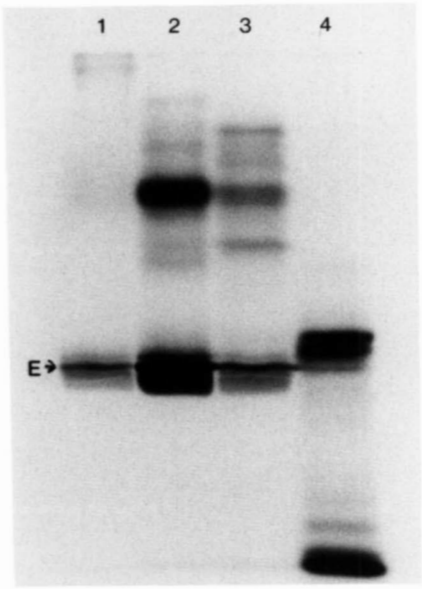


Fig. 8. SDS-PAGE analysis of media proteins separated by 10% agarose column chromatography, as shown in Fig. 7B. Aliquots of pooled, dialyzed and lyophilized column fractions 26-29 (lane 1), 30-33 (lane 2), 34-38 (lane 3) and 48-50 (lane 4) were resolved by SDS-PAGE and fluorographed.

incubated under identical conditions in the absence of cells did not change its elution characteristics.

SDS-PAGE analysis of the [^{35}S]methionine-labeled proteins in the apoE-containing fractions shown in Fig. 7B is presented in **Fig. 8.** A substantial portion (>80% by scanning) of the labeled protein eluting in the void volume of the column is apoE (lane 1). ApoE also represents a substantial amount of the media proteins eluting in the ascending and descending portions of the HDL_3 peak (lanes 2 and 3, respectively). Only a small fraction of the radiolabeled proteins eluting near the bed volume of the column is apoE. These observations confirm the results obtained by immunoprecipitation of apoE.

Over 90% of the apoE found in the void volume in panel A and in the first two apoE fractions of panel B could be reisolated in the d < 1.21 g/ml fraction upon ultracentrifugation (not shown). Conversely, the apoE found in the fractions corresponding to the delipidated rat apoE remained in the d > 1.21 g/ml fraction.

DISCUSSION

The cholesteryl ester mass of the cholesterol-loaded macrophages is involved in a cycle of continuous hydrolysis and re-esterification with an approximate half-life of 24 h (24). Inhibition of ACAT, the enzyme responsible for cholesterol esterification, breaks this cycle and, in the absence of a cholesterol acceptor in the media, results in progressive accumulation of free cholesterol within the cells. The rate of cholesteryl ester depletion and free cholesterol accumulation within these cells is consistent with the reported cholesteryl ester turnover in macrophage foam cells (24). The cholesteryl ester cycle can also be interrupted by incubating the cells with a cholesterol acceptor; the free cholesterol, localized primarily in the plasma membrane, is desorbed from the membrane and diffuses through the aqueous phase until it collides with HDL (25). The results of the present studies, based on mass measurements alone, are in agreement with this. ACAT inhibition results in a progressive accumulation of free cholesterol within the cells, much of it presumably accommodated in the plasma membrane. Addition of HDL_3 to these cells causes a significant increase in cholesterol efflux compared to cells with active ACAT, presumably due to increased free cholesterol pool available for efflux.

Studies by Basu, Goldstein, and Brown (26) have shown that cholesterol efflux and apoE secretion in resident mouse peritoneal macrophages are not coupled tightly: apoE is not essential for cholesterol efflux and vice versa. The observations made in the present studies, using TG-elicited macrophages, are in agreement: cholesterol loading, in absence of efflux resulted in a fivefold increase in media apoE accumulation and a twofold increase in apoE mRNA. The present experiments demonstrate, however, that cholesterol efflux accelerates apoE synthesis and secretion. This effect is apparently not mediated at the transcriptional level: apoE mRNA levels did not change in response to additional manipulations. Cholesterol efflux from macrophages was first stimulated by addition of HDL_3; it was than further accelerated by coincident inhibition of cellular ACAT. This resulted in virtual doubling of the rate of cholesterol efflux from the cells, over the same period of time (20% decrease vs. 35% decrease in cell cholesterol content), and a similar increase in the amount of apoE secreted. As would be expected, the accelerated rate of apoE secretion was accompanied by decreased cellular apoE content. Significantly, increasing the cellular free cholesterol content at the expense of cholesteryl esters, from 35% to 56% of total cellular cholesterol mass, in cholesterol-loaded cells, in the absence of cholesterol efflux, had no effect on apoE mRNA levels and rates of apoE synthesis. These observations are in contrast to the recent report that intracellular free cholesterol up-regulates apoE synthesis (27). Comparisons between these studies should be made carefully, since, in addition to a number of differences between resident and elicited macrophages, there are differences in the experimental design. Nevertheless, the present study suggests that, in addition to cellular cholesterol mass (free or esterified), free cholesterol movement from the cell into the media is an important promoter of apoE synthesis and secretion.

The regulation of the apoE gene expression in the murine macrophage may be regulated at several levels.

Stimulation with a nonimmunogenic agent such as thioglycolate induces a significant increase in the rate of apoE synthesis and secretion (14), possibly at the level of transcription. Cholesterol loading results in an additional increase in apoE mRNA levels. The observed differences in the fold stimulation of the message (twofold) and the fold stimulation in the rate of synthesis (~fivefold) is suggestive of translational regulation. This is further supported by the lack of additional changes in the levels of the message in the face of significant changes in the rates of total apoE accumulation under the various experimental conditions used in this study.

The mechanisms responsible for the acceleration of apoE secretion by cholesterol efflux remain speculative at this time. It is possible that at least some of the apoE secretion is mediated by HDL binding to the macrophage. Exposure of cholesterol-loaded macrophages to the ACAT inhibitor increased HDL binding twofold (L. Dory, unpublished observations). Increased HDL binding under these conditions may account for increased rate of apoE secretion (and increased cholesterol efflux). Alternatively, HDL_3 present in the media may bind the newly secreted apoE and thus diminish possible re-uptake by the cells. The binding of apoE by HDL_3 may represent a physiological phenomenon, much in the same way as is the removal of cellular cholesterol from the cells. Further studies are necessary to determine the precise nature of the underlying mechanism.

Column chromatography analysis of the macrophage-conditioned media indicates that in the absence of HDL_3 in the media, apoE is secreted as a large molecular weight complex, presumably in association with lipid. Indeed, over 95% of this material is of $d < 1.21$ g/ml (results not shown). Clearly, the size and the flotation characteristics of this fraction suggest the association of finite amounts of cholesterol and phospholipids with the newly secreted apoE. Similar observations were reported by Basu and coworkers (26), who reported the association of the macrophage-derived apoE with a discoidal particle of a mean density of 1.075 g/ml and a diameter of 180 Å (5). Discoidal, free cholesterol- and phospholipid-rich lipoprotein particles containing peripherally synthesized apoE were also isolated from canine prenodal peripheral lymph (11). It is reasonable to suggest that cholesterol esterification (by LCAT) may be necessary for further remodeling of these particles, and possibly for their association with other plasma lipoproteins. This notion is supported by the observation that similar particles, isolated from peripheral lymph of cholesterol-fed dogs, were transformed by the action of LCAT into larger, lower density lipoproteins resembling HDL_C (28).

A small but consistent portion of apoE is secreted by cholesterol-loaded macrophages in an essentially lipid-free form. The possibility that some of the apoE may dissociate from the large complex during the process of media concentration by ultrafiltration has not been eliminated.

Much of the increase in apoE secretion in the presence of HDL_3 in the incubation media appears associated with a remodeled HDL particle. Presumably, as a result of its interaction with the cholesterol-loaded cell, the HDL_3 increases in size and acquires apoE. A small but consistent fraction is also converted into a large lipoprotein, eluting in the void volume of the column. These observations are consistent with previous reports of HDL remodeling by exposure to macrophages (29).

The present experiments raise a number of important questions. While apoE secretion and cholesterol efflux can be dissociated under experimental conditions, there is a close relationship and interdependence between these two processes. The secretion of apoE in two or three different molecular forms (depending on the incubation conditions) raises the question of their metabolic fate. Sequential addition of other plasma components and enzymes, such as LCAT, may provide additional insight into the process of reverse cholesterol transport.

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