Expression of human apolipoprotein E but not that of apolipoprotein A-I by mouse C127 cells is associated with increased secretion of lipids in the form of vesicles and discs

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Abstract Transfected mouse mammary-derived cells (C127) expressing human apolipoprotein (apo) E (C127E) were used *a*) to determine whether the lipid-binding character of apoE is sufficient to promote its assembly with lipid to form lipoprotein-like particles when expressed by cells not normally expressing apolipoproteins; *b*) to characterize the secreted complexes in terms of morphology, size, and composition; and finally *c*) to determine whether apoE or apoA-I gene expression by these transfected cells has any effect on the levels and the profiles of the synthesized and secreted lipids. The findings of the present study demonstrate that: *a*) as determined by density gradient ultracentrifugation and gel filtration chromatography, about 20% of the secreted [^{35}S]methionine-labeled apoE expressed by C127E cells is lipid-associated. *b*) Negative-stain electron microscopic analysis of the lipid-protein complexes recovered in the lipoprotein fractions (d < 1.21 g/ml) revealed that ~13% of the total population of particles were discs (16 ± 5 nm mean diameter and 4–6 nm thick), resembling nascent high density lipoproteins (HDL). The majority of the particles however (>82%) appeared vesicular with varying diameters (48 ± 40 nm mean diameter). The discoidal and the vesicular appearance of the particles secreted by C127E cells is consistent with the composition of lipids. These consisted mostly of surface lipids, phospholipids (45 ± 18%), diacylglycerols (36 ± 17%), and free cholesterol (17 ± 7%) (by weight). *c*) Expression of apoE by C127E cells was associated with an increased release of [^{35}S]methionine-labeled protein and [^{3}H]glycerol-labeled lipid (3- to 5- and 4- to 8-fold, respectively) compared to nontransfected C127 cells. Expression of mutant apoE or normal apoA-I, however, was not associated with increased release of the major lipid classes compared to the parent C127 cells, strongly suggesting that this character of C127E cells is specific to apoE expression. The release of lipids by C127E cells could be reduced considerably by the addition of the metabolic inhibitors, colchicine or cycloheximide (10 and 1 μM, respectively), suggesting that lipid release by C127E cells is an active process requiring both protein synthesis and functional secretory mechanisms. Taken together the findings suggest that apoE expression by C127 cells promotes the formation of nascent discoidal lipoprotein-like particles and enhances the release of vesicular lipids, possibly by promoting shedding of cell plasma membrane fragments.—**Herscovitz, H., D. Gantz, A. M. Tercyak, V. I. Zannis, and D. M. Small.** Expression of human apolipoprotein E but not that of apolipoprotein A-I by mouse C127 cells is associated with increased secretion of lipids in the form of vesicles and discs. *J. Lipid Res.* 1992. **33:** 791–803.

Supplementary key words lipoproteins • apoE-producing cell lines • vesicles • discoidal lipoproteins

Apolipoprotein (apo) E is a protein constituent of a variety of lipoproteins including chylomicrons and their remnants, very low density lipoproteins (VLDL), β-VLDL, intermediate density lipoproteins (IDL), and a subclass of high density lipoproteins (HDL) (called HDL with apoE) (1). The presence of apoE on surfaces of lipoproteins plays a key role in their metabolism, since it mediates their uptake and subsequent catabolism by directing them to specific high affinity receptors that recognize apoE as their ligand. Two types of receptors bind apoE: the LDL (B,E) receptor expressed on hepatic and extrahepatic cells (2–6) and the chylomicron remnant (apoE) receptor thought to be functional only on hepatic cells (7, 8). Variants of apoE, defective in binding to lipoprotein receptors, lead to a substantial accumulation of remnant lipoproteins in the plasma and are associated with type III hyperlipoproteinemia (9–11).

Abbreviations: apo, apolipoprotein; HDL, high density lipoproteins; LDL, low density lipoproteins; VLDL, very low density lipoproteins; EM, electron microscopy; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; DMPC, dimyristoylphosphatidylcholine; PL, phospholipids; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; PI, phosphatidylinositol; DAG, diacylglycerols; TAG, triacylglycerols; CE, cholesteryl esters; FCS, fetal calf serum.

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ApoE, a 299-amino acid protein of an apparent molecular weight of 34,200 Da (12) is synthesized and secreted primarily by the liver (13, 14). However, many other tissues also synthesize and secrete apoE (15–20) and can contribute between 20 and 40% of the total plasma apoE pool (18–20).

ApoA-I is the major protein constituent of human HDL and is thought to play a major role in the reverse cholesterol transport from peripheral cells to the liver (21). ApoA-I also serves as activator of the enzyme lecithin:cholesterol acyl transferase (22). Unlike apoE, apoA-I is synthesized and secreted primarily by the liver and the intestine (23).

The mechanisms involved in the processing of newly synthesized apolipoproteins such as apoE and apoA-I to form lipoproteins are not well understood. Presently, it is not clear whether the association of these proteins with lipids occurs prior to, concurrent with, or after their secretion into the plasma. Both apolipoproteins can exist either lipid-associated or lipid-free (24). Due to the existence of sequences predicted to form amphipathic α helices (25), it is expected that their preferred environment is the lipid surfaces of lipoproteins (24). Interaction of these proteins with lipids results in their conformational stabilization (26, 27). Therefore, if they are secreted unbound to lipid they could readily associate with appropriate membrane lipids or with preexisting lipoproteins (21) to achieve higher energy of stabilization (24).

The use of hepatic or intestinal cells to elucidate the mechanisms responsible for the assembly of these proteins with lipids has the disadvantage that these cells synthesize a variety of apolipoproteins and process large amounts of lipids at the same time. To simplify the problem, various investigators have made use of in vitro expression systems that utilize mammalian cells that do not normally express apolipoproteins so that single apolipoproteins are expressed (28–30). In the present study we attempted to determine whether expression of apoE or apoA-I can induce significant changes in lipid synthesis and secretion and whether the expressed apolipoproteins are able to form lipoprotein-like particles. The data show that expression of apoE, unlike that of apoA-I, by transfected mouse cells leads to enhanced secretion of lipids, about half of which are associated with apoE. This association between apoE and lipid may occur both intra- and extracellularly.

MATERIALS AND METHODS

Bovine serum albumin (BSA), rabbit anti-goat purified antibodies, gold- (5 nm) and peroxidase-conjugated, and IgGsorb, were purchased from Sigma (St. Louis, MO); goat anti-human apoE was obtained from Atlantic Antibodies (Scarborough, ME); human apoE3 was a generous gift from Dr. Karl Weisgraber of the Gladstone Foundation Laboratories (San Francisco, CA). Dulbecco's modified Eagle's medium (DMEM), fetal calf serum (FCS), trypsin-EDTA, and glutamine were purchased from Gibco Laboratories (Grand Island, NY); Sephacryl S-400 SF and S-1000 SF were purchased from Pharmacia LKB (Piscataway, NJ); concentrator Centricon was obtained from Amicon (Danvers, MA) and concentrator Centricell was obtained from Polysciences (Warrington, PA); [2-^{3}H]glycerol (0.5–1.0 Ci/mmol) was purchased from Amersham (Arlington Heights, IL). [^{35}S]methionine (800–1000 Ci/mmol) and enhancer, EN[^{3}H]ANCE, were purchased from Du Pont-New England Nuclear (Boston, MA).

Cell lines and cell cultures

The parent C127 cell line was derived from a mouse mammary carcinoma (31). The selection of C127 cell clones, expressing the normal and mutant apoE (32), and normal apoA-I genes (33), designated C127E, C127Ed, and C127A-I, respectively, has been described. Freshly thawed (up to the tenth passage) C127 cells and transfected clones were maintained in DMEM supplemented with 10% (v/v) FCS in a humidified 5% CO_2–95% air incubator at 37°C.

In all experiments, cells grown to 80–90% confluency were pre-incubated for 5 h in serum-free (SF) DMEM followed by incubation either in SF-DMEM supplemented with [2-^{3}H]glycerol or in methionine-free/SF-DMEM supplemented with [^{35}S]methionine and 5 μM methionine to label lipids or proteins, respectively. Incubation time was carried out for up to 40 h. This incubation time was selected for most experiments for convenience, since it was found that only a small fraction of the labeled lipids was secreted by 24-h incubation. The major portion of the labeled lipids was apparently secreted between 24 h and 45 h. This presumably reflects an increase in the specific radioactivity of the lipids destined for secretion as the mass of lipids accumulating in the media increased in a linear fashion (data not shown). Trypan Blue exclusion showed that $>$ 85% of the cells were viable after 24 or 48 h. Also, glucose consumption levels were very similar after 24 or 48 h, suggesting that the cells were metabolically active at least up to 48 h.

To harvest the cells, monolayers were rinsed with cold PBS, trypsinized with trypsin-EDTA, and collected by centrifugation followed by lipid extraction. Alternatively, cells were lysed in 2% SDS, adjusted to 0.05% SDS, 0.02% sodium azide, and 1 mM PMSF in PBS, pH 7.4, and used for total protein analysis and immunoprecipitations. The media were collected and centrifuged 2 $\times$ 30 min at 1000 *g* at 4°C to remove cellular debris, adjusted to 3 mM EDTA, 1 mM PMSF, and 0.02% sodium azide at pH 7.4, and concentrated 20- to 40-fold using concentrators of 30,000 molecular weight cutoff.

To separate total lipid-bound proteins, the density was adjusted to 1.215 g/ml with solid KBr and the samples

were subjected to ultracentrifugation at 40,000 rpm in a Beckman SW-41 rotor at 15°C for 48–72 h. Alternatively, conditioned media were subjected to density gradient ultracentrifugation in a discontinuous gradient (34). Ultracentrifugation as described above was for 24 h. Fractions were collected from the top of the tubes. Densities from a blank tube were estimated by refractometry (American Optical Corporation).

Gel filtration chromatography

Samples containing apoE in PBS, pH 7.4, containing 0.02% sodium azide and 1 mM EDTA were applied to Sephacryl S-400 or S-1000 columns, and eluted at a flow rate of 10 ml/h in the same buffer at room temperature. Elution was monitored by absorbance at 280 nm and by measurements of radiolabeled lipids and proteins.

SDS-polyacrylamide gel electrophoresis (PAGE) and immunoblotting

Ten percent SDS-PAGE was performed as described (35). Gels were fluorographed at −70°C. The separated protein bands were excised from the gels and incubated in 30% H_2O_2. The radioactivity was determined by liquid scintillation spectrometry. For immunoblotting, proteins separated by 10% SDS-PAGE were transferred to a nitrocellulose filter at 100 mA for 16–18 h at 4°C (36). ApoE was detected by incubation with goat anti-apoE antiserum followed by colloid gold-conjugated rabbit anti-goat IgG and silver enhancement.

Immunoprecipitation of apoE

Cell lysates were suspended in PBS, pH 7.4, containing 5 mg/ml BSA, 0.5% SDS, 1% Triton X-100 and 1% sodium deoxycholate. IgGSorb was added to cell lysates and the mixtures were incubated at 4°C for 1 h. The supernatants obtained after centrifugation were then incubated overnight at 4°C with goat anti-human apoE antiserum diluted in the same buffer. IgGSorb was then added and mixtures were further incubated for 1 h at 4°C. Washed immune complexes were either suspended in water and counted as described above or separated first on 10% SDS-PAGE, and protein bands were counted as described above. Non-immune serum and C127 cells served as negative controls. ApoE was also precipitated from the medium as described above except that SDS, Triton, and deoxycholate were omitted from the buffer.

Competitive enzyme-linked immunosorbent assay (ELISA)

The mass of apoE was determined by competitive ELISA which was carried out essentially according to Engvall (37). Horseradish-conjugated rabbit anti-goat IgG was used for detection.

Electron microscopy

Two different negative staining protocols were used to prepare samples: *a*) "Drop method": 8 μl sample was pipetted onto glow-discharged carbon-coated 300-mesh grids followed by a drop of 1% phosphotungstic acid, pH 7.4. *b*) "Diffusion (mica) method": carbon film from freshly coated mica was sequentially floated on 50-μl aliquots of sample and stain (38). Samples were analyzed by Hitachi HU-11C microscope. Calibration was carried out by carbon grating replica with 2160 lines/mm (Ernest F. Fullam Inc., Latham, NY).

Radioactive analyses

Radiolabeled lipids were extracted with chloroform–methanol 1:2 (39). The chloroform phase was washed with 0.5 N acetic acid in saline, and the extracted lipids were dried under N_2 and redissolved in a small volume of chloroform–methanol 1:1. Aliquots were mixed with carrier standards and separated by thin-layer chromatography (TLC) on silica gel G plates developed in hexane–ethyl ether–acetic acid 70:30:1 or chloroform–methanol–acetic acid–water 50:30:8:4 to separate neutral or polar lipids, respectively. Lipid classes were visualized by exposure of plates to iodine vapors, scraped into scintillation vials, and counted as described above. The radioactivity associated with total proteins was determined after precipitation of lysates and culture media with cold 10% trichloroacetic acid.

Chemical analyses

Phospholipids (PL) were determined as inorganic phosphate (40); tri- and diacylglycerols (TAG and DAG, respectively) were separated by TLC and were determined thereafter as glycerol liberated after alkaline hydrolysis (5% alcoholic KOH) using a kit from Sigma. Cholesterol and esterified cholesterol (CE) were determined using cholesterol oxidase (41). Total cellular and secreted proteins were determined as described (42) using BSA as a standard.

Statistical analysis

Statistical analysis of unpaired samples was performed by a software package RS/1 (BBN Research Systems). Differences regarding lipid synthesis and secretion between the parent cell line and the transfected cell clones were considered significant at the 95% confidence level.

RESULTS

Fractionation of conditioned media by density gradient ultracentrifugation and gel filtration chromatography

Density gradient ultracentrifugation. To determine whether apoE expressed by C127 cells is secreted associated with

lipid, media conditioned by C127E cells in the presence of [^{35}S]methionine or [^{3}H]glycerol were fractionated by density gradient ultracentrifugation. Typical density profiles of labeled lipids, proteins, and apoE are illustrated in **Fig. 1** (panels A, B, and C, respectively). As calculated from Fig. 1C, a fraction, ~25% of the secreted ^{35}S-labeled apoE, was recovered in the combined lipoprotein fractions (d < 1.21 g/ml), suggesting that this fraction of apoE is lipid-associated. The major portion, however, was apparently lipid-poor as it was recovered from d > 1.21 g/ml. The major fraction of ^{3}H-labeled lipids was recovered from d < 1.21 g/ml having a peak density of 1.15 g/ml and a mean of 1.12 g/ml (Fig. 1A). It was found that 40–50% of the total secreted ^{3}H-labeled lipids in unfractionated media were associated with apoE as determined by their precipitation with antibodies directed against apoE.

A comparison of the density profiles of labeled lipid and proteins released by C127E cells to those released by the nontransfected C127 cells showed that C127E cells secreted considerably more labeled lipids (~6-fold; range 4–8) and labeled proteins (~4-fold; range 3–5) than the parent C127 cells (Fig. 1, panels A and B, respectively).

To determine whether the increased release of lipids and proteins by C127E cells is an active process, these cells were labeled with either [^{35}S]methionine or [^{3}H]glycerol for 18 h in the presence of metabolic inhibitors, cycloheximide (1 μM) or colchicine (10 μM). It was found that cycloheximide, concurrently with protein synthesis inhibition, reduced the release of ^{3}H-labeled lipids to 30 ± 3% of control levels. The total synthesis of ^{3}H-labeled lipids, however, was unaffected by cycloheximide. Colchicine reduced the release of both labeled proteins and lipids to 44 ± 4% and 40 ± 3%, respectively, of control levels without affecting the levels of synthesis of either lipids or proteins. These findings, therefore, strongly suggest that the release of lipids and proteins by C127E cells does not result from passive release of intracellular components due to dying cells but is rather an active process requiring both synthesis of protein and a functional transport system. Similar results were also obtained for the control C127 cells (data not shown).

Gel filtration chromatography on Sephacryl columns. Gel filtration chromatography was used both to determine further the amount of lipid-bound apoE and to characterize the lipid-protein complexes released by the cells in terms of size and composition. Conditioned media harvested from C127E cells after a 40-h labeling period were fractionated on Sephacryl S-400 column (**Fig. 2**). The column was calibrated with isolated lipoprotein fractions to estimate the approximate size of the particles. Shown in Fig. 2A are typical elution profiles of labeled lipids and proteins. The major fraction, 65–70% of the total secreted ^{3}H-labeled lipids, eluted in a single peak with the void volume. However, only 15–20% of the total secreted ^{35}S-

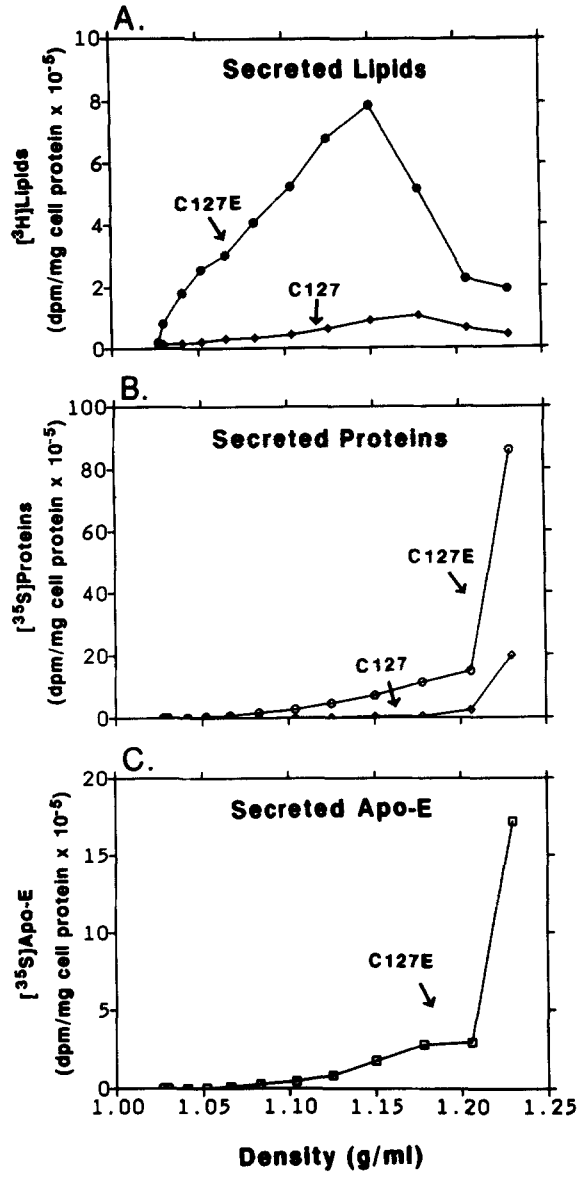

Fig. 1. Density distribution of radiolabeled lipids and proteins. Subconfluent C127 and C127E cells were labeled for 40 h with [^{3}H]glycerol or [^{35}S]methionine. The media were harvested, concentrated, and subjected to discontinuous density gradient ultracentrifugation. Fractions were collected and the following analyses were performed. Total ^{35}S-labeled proteins were determined after precipitation with 10% trichloroacetic acid. ^{35}S-labeled apoE was determined after immunoprecipitation with anti-human apoE antibodies. ^{3}H-labeled lipids in chloroform extracts were determined as described under Materials and Methods. A typical experiment is shown. A: ^{3}H-labeled lipids in C127E (closed circles) and C127 (closed diamonds) media. B: ^{35}S-labeled proteins in C127E (open circles) and C127 (open diamonds) media. C: ^{35}S-labeled apoE in C127E medium.

labeled apoE co-eluted with the lipids in the void volume (Fig. 2B) as determined by immunoprecipitation of apoE from pooled fractions (shown in panel A). Therefore this fraction of apoE was probably lipid-associated. SDS-PAGE showed that the void volume fractions contained apoE (identified also by Western blotting) as the major band

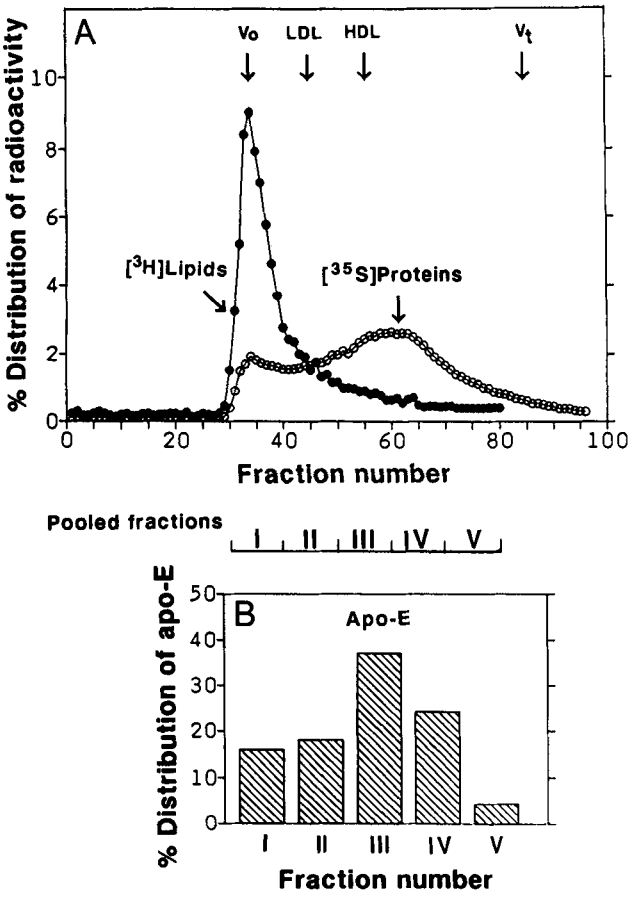

Fig. 2. Fractionation of medium conditioned by C127E cells on Sephacryl S-400 column. C127E cells were radiolabeled as described in Fig. 1. Media were harvested, concentrated, and applied to the S-400 column. The radioactivity associated with lipids and proteins in each fraction was determined as described in the legend of Fig. 1. A: Elution pattern of ^{3}H-labeled lipids and ^{35}S-labeled proteins (closed and open symbols, respectively) expressed as percent of the total radioactivity recovered from the column. B: ^{35}S-labeled apoE immunoprecipitated from the pooled fractions (designated by Roman numerals as follows: pooled fractions I = fractions 31–40, pooled fractions II = fractions 41–50, pooled fractions III = fractions 51–60, pooled fractions IV = fractions 61–70, and pooled fractions V = fractions 71–80) expressed as percent of total radiolabeled apoE recovered from the column.

(data not shown) and accounted for 30–35% of total proteins in these fractions. The void volume fractions represent lipid/protein complexes as well as lipid particles having diameters of 25 nm or more. The immediate post-void volume fractions could contain smaller particles. However, only a small fraction of the labeled lipids eluted with these fractions, thus indicating that only a small number of particles having diameter < 25 nm were present in these fractions. This assumption was verified by electron microscopic analysis of these fractions which showed no evidence for the presence of small particles (data not shown). Some radiolabeled proteins of similar distribution and composition were obtained from the analysis of media derived from C127-cells, except that the percentage of radiolabeled lipid-bound proteins is much smaller (approximately 5–6% of total radiolabeled proteins).

To separate the lipid-protein complexes released by C127E cells, the void volume fractions of Sephacryl S-400 column (fractions 30–40 in Fig. 2A), were applied onto a Sephacryl S-1000 column. Radiolabeled proteins and lipids displayed similar elution patterns (**Fig. 3A**). Approximately 10–15% of total radiolabeled lipids and proteins co-eluted with the void volume of the column. The

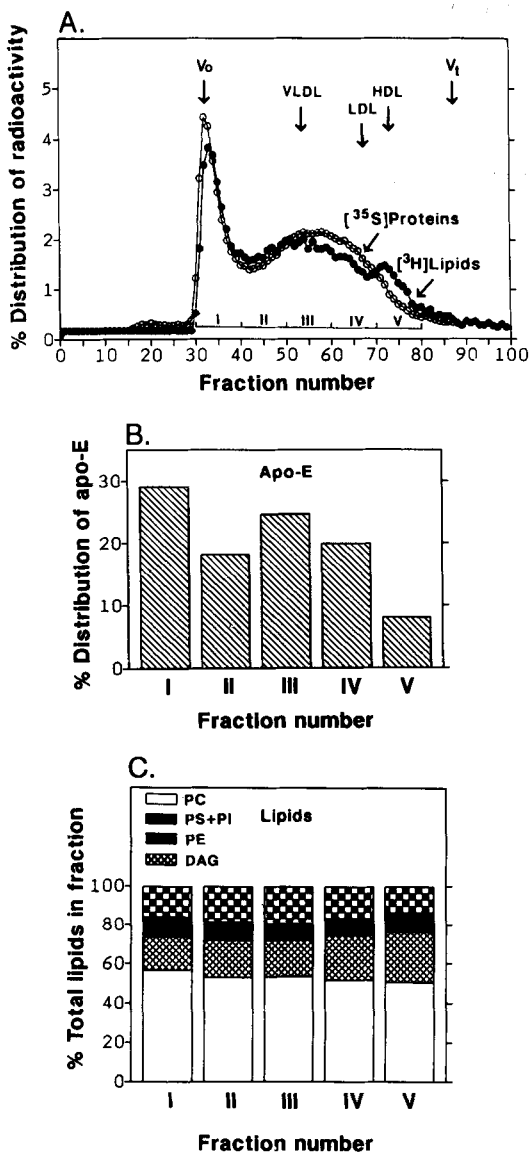

Fig. 3. Fractionation on a Sephacryl S-1000 column. C127E cells were labeled and processed as described in Fig. 2. Fractions 31–40 eluted from a Sephacryl S-400 column (see Fig. 2) were pooled and applied onto an S-1000 column. The figure shows the patterns of radioactivity expressed as percent of the total radioactivity recovered from the column. A: Elution patterns of ^{3}H-labeled lipids and ^{35}S-labeled proteins (closed and open symbols, respectively). B: Elution pattern of ^{35}S-labeled apoE immunoprecipitated from the pooled fractions (designated by Roman numerals). C: Lipid composition of the same pooled fractions determined by the radioactivity associated with the lipid classes obtained following separation by TLC as described under Materials and Methods.

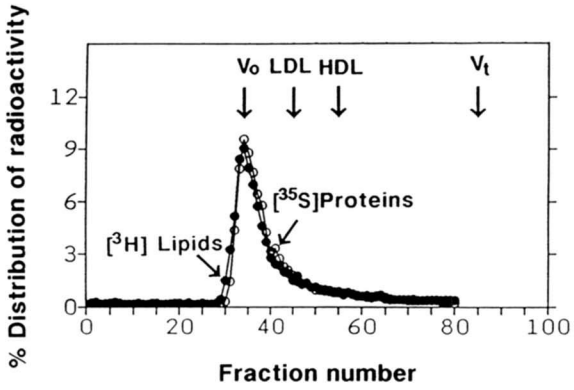

Fig. 4. Fractionation of the d < 1.21 g/ml fraction of culture media of C127E cells on a Sephacryl S-400 column. C127E cells were labeled and ultracentrifuged at d 1.215 g/ml. The top fraction (d < 1.215 g/ml) was removed, dialyzed, and fractionated on an S-400 column.

majority of the radioactive lipids and proteins eluted later in the run and displayed a very broad peak. The distribution of apoE present in the pooled fractions (as indicated by Roman numerals) (Fig. 3B) was similar to that of the total secreted radiolabeled proteins (Fig. 3A). The fractions in the void volume correspond to particles larger than 100 nm in diameter or to complexes with irregular morphology. The great majority of particles appeared heterogeneous in size and had effective diameters greater than human LDL. The composition of lipids eluted in the different fractions was very similar (Fig. 3C). This indicates that the size heterogeneity is not the result of major compositional differences. Similar composition across the wide size range is consistent with the existence of vesicular particles of variable size and similar relative composition.

The radiolabeled lipoprotein fraction of C127E medium (d < 1.21 g/ml) was also fractionated on a Sephacryl S-400 column. As shown in **Fig. 4** the radiolabeled lipids and proteins co-eluted with the void volume. The ratio of lipid to protein was constant and no additional peaks were detected. Thus it appears that most lipid/protein complexes released by C127E cells are larger than 25 nm effective diameter, and smaller complexes probably represent a minor fraction of the total secreted lipids.

Morphology, size, and composition of lipid/protein complexes

The d < 1.21 g/ml fraction obtained by ultracentrifugation of C127E conditioned medium was examined by electron microscopy (EM) after negative staining to characterize further the morphology and the size of lipid/pro-

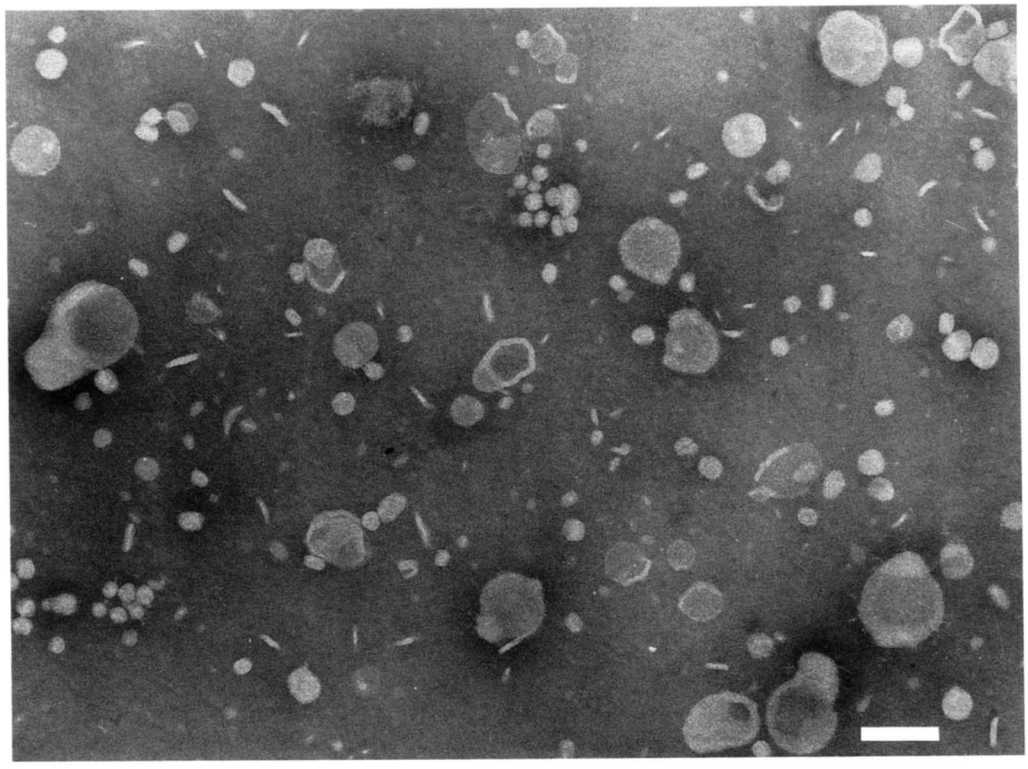

Fig. 5. Electron micrograph of negatively stained samples obtained from the d < 1.21 g/ml fraction C127E culture media. Subconfluent C127E cells were grown for 40 h in serum-free DMEM. Media pooled from ten 100-mm diameter dishes were concentrated and centrifuged at d 1.215 g/ml. The top d < 1.21 g/ml fraction was dialyzed against PBS and mounted onto carbon surfaces by the "Diffusion method" as described under Materials and Methods. The bar indicates 100 nm. The magnification factor is 102,500.

tein complexes. This analysis showed a rather heterogeneous population of particles (**Fig. 5**) that were categorized arbitrarily into several groups as follows: *a*) small circular particles ranging from 4 to 12 nm in diameter; *b*) linear or fusiform ranging from about 8 to 28 nm in length and from 4 to 24 nm in thickness; *c*) larger particles of roughly circular morphology ranging from 14 to more than 150 nm in diameter; and *d*) particles with irregular morphology. The latter two categories consisted mostly of particles that appeared vesicular by negative staining. The overall size distribution of the particles is shown in **Fig. 6**. The mean diameter of the circular particles is 48 ± 40 nm (calculated from the data shown in Fig. 6A). The shape of the linear and fusiform particles was studied by the "diffusion (mica) method" (38) which facilitates the adherence of particles on edge at variable angles. Thus discs or collapsed vesicles adhering with their discoidal plane normal to the grid will appear as linear particles of constant thickness equivalent to the thickness of the disc or vesicle. Discs or vesicles adhering at angles not normal to the grid will appear as fusiform or ellipsoidal shapes. The thickness of all linear and fusiform particles is shown in Fig. 6B. The thinnest particles (4–6 nm) were linear whereas the thicker particles were both linear and fusiform. As the thickness of a single bilayer formed by phospholipids in water is about 4–6 nm (43), we concluded that those linear particles of approximately 4–6 nm in thickness must be single bilayered discs. This group of particles had a mean diameter of 16 ± 4.6 nm (calculated from the data shown in Fig. 6C). Linear particles of 8 nm and larger in thickness presumably represent collapsed vesicles, some of which had a thin black line that results from stain penetration. Based on measurements obtained from two separate experiments, the small circular particles and single bilayered particles (discs) comprised approximately 5% and 13%, respectively, of the total population of particles, whereas the larger vesicular particles comprised approximately 80% of total particles. This distribution is consistent with the data obtained from fractionation of culture media on Sephacryl S-400 and S-1000 columns that indi-

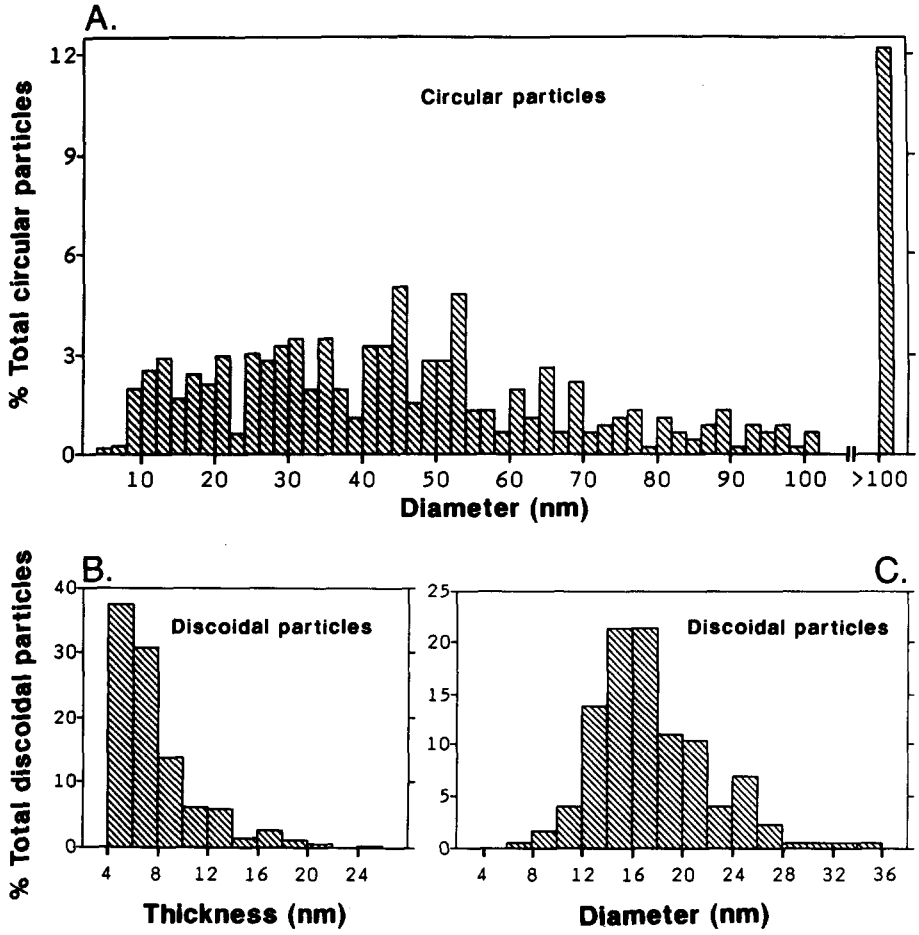

Fig. 6. Diameter and thickness distribution of particles present in the d < 1.21 g/ml C127E media. Particles shown in Fig. 5 were classified and sized. A: Size distribution of total circular particles. The last bar represents particles of a diameter between 100 and 200 nm. B: Thickness distribution of total discoidal particles. C: Size distribution of discoidal particles of 4–6 nm thickness.

TABLE 1. Chemical composition of lipid-protein complexes secreted by C127E and C127 cells

Composition	C127E (n = 7)	C127 (n = 6)
	total μg/mg cell protein	
Lipid	52.0 ± 15.6	10.9 ± 3.2
Protein	22.3 ± 6.7	8.6 ± 2.6
	wt %	
Protein	30.0 ± 10.0	44.2 ± 12.0
Phospholipids	31.3 ± 12.5	31.5 ± 14.7
Diacylglycerols	25.3 ± 11.8	11.6 ± 7.4
Cholesterol	11.4 ± 4.9	12.7 ± 3.8
Triacylglycerols	2.0 ± 1.0	<1
Cholesteryl esters	<1	nd

Media from C127E and C127 control cells were collected after a 40-h incubation period. Lipid-protein complexes were recovered from the d < 1.21 g/ml after ultracentrifugation at d 1.215 g/ml. Lipids and proteins were determined as described in Materials and Methods. The data represent means ± SEM; nd, not detected.

cated that most of the lipid radioactivity was associated with particles of a diameter larger than 25 nm (Figs. 2 and 3). These data strongly suggest that while most of the apoE in C127E media is lipid-free, a small fraction is associated with complexes of vesicular and discoidal nature. Similar analysis of samples derived from media obtained from control C127 cells showed that these cells secrete small amounts of particles with general features similar to those observed in C127E media (data not shown). However, the relative abundance of the discoidal and small circular particles was much less and comprised only 2% and 3%, respectively, of the total secreted particles. Since the absolute secretion of lipid is much less in these cells, C127E cells actually secrete 17–34 times more discoidal particles.

The composition of particles recovered in the lipoprotein fractions of C127E and C127 media is shown in **Table 1**. Lipid analysis showed the virtual absence of core lipids, TAG and CE. These observations are consistent with EM analysis which suggested a vesicular or discoidal nature of the secreted particles. The mass of DAG comprised 30% of total secreted lipids. DAG could be generated intracellularly prior to their secretion or extracellularly by the hydrolytic activity of lipases that could either be secreted into the culture medium or could reside on the cell plasma membrane surfaces. Control experiments that were designed to search for such lipases failed to show any such activity. Thus, DAG are probably generated intracellularly. The total mass of the lipids secreted by C127E cells was about 5-fold higher than that secreted by the parent C127 cells, consistent with the data shown in Fig. 1A.

Radioactive lipid synthesis and secretion by C127 and transfected C127 clones

As shown in Fig. 1A, C127E cells secreted several-fold more radiolabeled lipids than the parent C127 cells. To determine whether the expression of apoE is specifically responsible for these differences or whether they result from the expression of any apolipoprotein or even from the transfection process itself, we compared the lipid profiles obtained from C127E cells to those obtained from C127 cells expressing either apoA-I (C127A-I) or a mutant apoE gene (C127Ed). This mutation is a splicing mutation resulting in the expression of two small fragments of apoE that accumulate in the cells. All four cell lines were labeled with [^{3}H]glycerol and the resulting intracellular and secreted lipid profiles were compared. This analysis showed that the relative abundance of most of the newly synthesized intracellular lipids were very similar in all four cell lines tested (**Table 2**), except for a significant elevation ($P < 0.002$) in the relative abundance of TAG in C127Ed cells. The analysis of the secreted lipids showed that C127E cells secreted a significantly higher content of labeled phosphatidylcholine (PC) ($P < 0.05$) compared to the parent C127, C127Ed, or C127A-I cells (Table 2), while C127Ed cells secreted a higher content of labeled TAG ($P < 0.02$). The relative abundance of the other lipid classes, however, was very similar in all four cell lines.

TABLE 2. Relative abundance of newly synthesized lipids in cells and media obtained from C127 cells and transfected clones

	Cells				Medium			
Lipid	C127E	C127Ed	C127A-I	C127	C127E	C127Ed	C127A-I	C127
DAG	4.4 ± 0.5	5.8 ± 0.8	4.7 ± 0.6	6.7 ± 2.0	20.7 ± 1.4	17.3 ± 3.9	22.9 ± 7.8	23.9 ± 1.5
TAG	8.1 ± 1.4[a]	13.8 ± 0.1[b]	7.1 ± 0.7[c]	3.7 ± 1.0	3.8 ± 1.0	16.8 ± 3.2[a]	6.1 ± 3.5	3.5 ± 1.2
LPC	0.4 ± 0.1	0.4 ± 0.2	0.2 ± 0.1	0.9 ± 0.4	0.8 ± 0.2	4.0 ± 1.8	0.6 ± 0.1	4.6 ± 2.1
SPH	0.3 ± 0.2	0.6 ± 0.5	0.2 ± 0.2	0.8 ± 0.5	1.1 ± 0.3	1.9 ± 0.7	0.9 ± 0.4	4.7 ± 2.4
PC	56.7 ± 1.7	51.0 ± 2.5	54.9 ± 5.5	57.5 ± 1.8	47.5 ± 4.9[a]	34.6 ± 7.2	31.8 ± 14.7	32.6 ± 7.5
PS + PI	16.8 ± 1.2	15.9 ± 0.5	17.5 ± 1.7	17.3 ± 1.8	18.0 ± 1.8	15.1 ± 2.5	24.2 ± 7.4	18.8 ± 3.3
PE	13.2 ± 1.5	13.0 ± 2.8	15.2 ± 3.7	13.0 ± 1.7	12.0 ± 2.1	10.3 ± 3.0	13.1 ± 1.0	11.8 ± 1.8

Subconfluent cells were labeled with [^{3}H]glycerol for 40 h. Total radioactivity was determined as described in Materials and Methods. The data represent % of total radioactive lipids (determined as dpm/mg cell protein). Abbreviations: DAG, diacylglycerols; TAG, triacylglycerols; LPC, lysophosphatidylcholine; SPH, sphingomyelin; PC, phosphatidylcholine; PS, phosphatidylserine; PI, phosphatidylinositol; PE, phosphatidylethanolamine.
Significantly different from C127 cells: [a], $P < 0.02$; [b], $P < 0.001$; [c], $P < 0.05$.

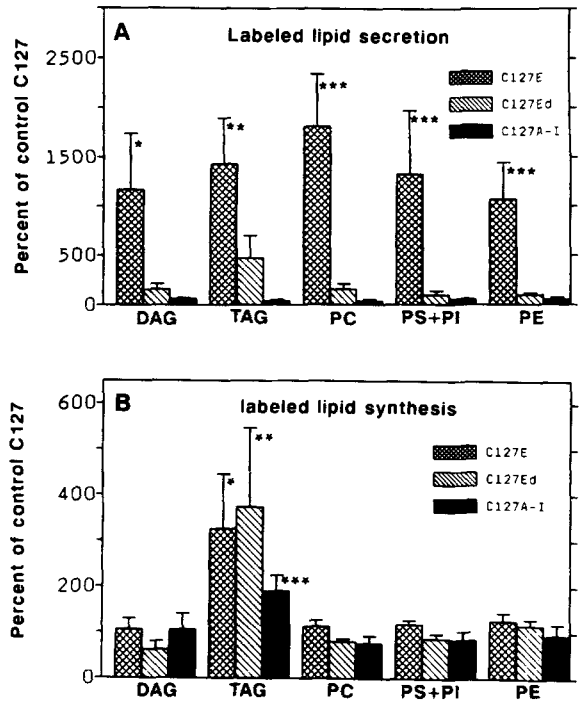

Fig. 7. Synthesis and secretion of radiolabeled lipids. C127 cells and transfected C127 clones were incubated as described in Table 2. Lipid levels calculated in dpm/mg cell protein were expressed as percent of lipids in control C127 cells. A: Total secretion of labeled lipids (* $P < 0.007$, ** $P < 0.002$, *** $P < 0.00005$). B: Total synthesis of labeled lipids (* $P < 0.0005$, ** $P < 0.08$, *** $P < 0.013$).

The synthesis and the secretion levels of radiolabeled lipids measured for C127E, C127Ed, and C127A-I and expressed as percent of control C127 cells are illustrated in **Fig. 7**. C127E cells secreted significantly larger amounts of radiolabeled lipids, both neutral lipids (DAG $P < 0.007$, TAG $P < 0.002$), and polar lipids ($P < 0.00005$), compared to the parent C127 and to the transfected clones, C127Ed and C127A-I (panel A). These observations suggest that increased secretion of lipids by C127E cells is specifically associated with the synthesis and secretion of normal apoE protein. Total lipid synthesis, however, appeared similar in all cell lines except for a significant elevation in synthesis levels of TAG by all three transfected clones (panel B) compared to C127 control cells, suggesting perhaps that the expression of apolipoprotein genes in general augments the synthesis of TAG.

Effect of extracellular apoE on lipid synthesis and secretion by C127 cells

The data shown in Fig. 7 indicated that apoE is responsible for the significant increase in lipid secretion by C127E compared to the parent C127 and other transfected C127 clones. ApoE could promote increased secretion of lipids during its synthesis as a result of intracellular lipoprotein assembly. Alternatively, the secreted lipid-poor apoE could bind to the cell plasma membranes, and facilitate the release of membranous components. To test the latter possibility, we labeled the parent C127 cells with [^{3}H]glycerol in the presence of increasing concentrations of apoE for various lengths of time and determined the synthesis and the secretion levels of radiolabeled lipids. In addition, we assessed the possible association of the extracellular apoE with lipids secreted by C127 cells by determining the amount of radioactive lipids immunoprecipitable with anti-human apoE antibodies. **Table 3** shows that under these experimental conditions a concentration-dependent increase in the secretion of radiolabeled lipids occurred. Thus, after a 40-h incubation period in the presence of 10 μg/ml apoE (which is comparable to the concentrations of apoE secreted by C127E cells following the same incubation period), a 2-fold increase in lipid secretion was observed. The extracellular

TABLE 3. Effect of lipid-free apoE on the synthesis and secretion of lipids by C127 cells

Additions	Incubation Time	Total Synthesized Lipids	Total Secreted Lipids	Lipids in Medium Immunoprecipitated with Anti-Human ApoE Antibodies
	h	*dpm/mg cell protein* × 10^{-3}		%
None	20	2,121	39	0
ApoE				
3 μg/ml	20	2,420	39	12
7 μg/ml	20	2,402	57	18
10 μg/ml	20	2,260	57	27
None	40	4,315	137	
ApoE				
3 μg/ml	40	5,110	141	13
7 μg/ml	40	5,110	183	20
10 μg/ml	40	5,432	240	30

C127 cells were labeled with [^{3}H]glycerol in the presence or absence of apoE purified from human plasma for the indicated time periods. Total radioactive lipids were extracted as described in Materials and Methods. Lipids bound to apoE were estimated by immunoprecipitation of the culture medium with anti-human apoE antibodies. Data represent the average of duplicate dishes.

apoE also showed a concentration-dependent association with the nascent lipids; thus between 12 and 30% of the total secreted lipids associated with the added apoE (Table 3). C127E cells, however, displayed a greater increase (4- to 8-fold) in lipid secretion compared to C127 cells, and approximately 40–50% of total lipids secreted by C127E cells were immunoprecipitable with anti-human apoE. The association of apoE with lipids secreted by C127 cells did not require the presence of the cells as apoE incubated for 20 h in medium conditioned by the parent C127 cells (for 40 h) allowed for immunoprecipitation of 28% of the total secreted lipids. Thus, lipid secretion by C127E cells is promoted by apoE synthesis and is enhanced by the accumulation of the nascent apoE in the culture media.

Formation of lipid-rich complexes by DMPC multilamellar vesicles and lipid-poor apoE present in C127E culture media

We tested the ability of nascent lipid-poor apoE secreted by C127E cells to form lipid-rich complexes with exogenous phospholipids. For this analysis the d > 1.21 g/ml fraction obtained by ultracentrifugation of the medium conditioned by C127E cells was mixed with DMPC multilamellar vesicles at the melting temperature of the acyl chains of DMPC, 24°C, at a DMPC–apoE weight ratio, respectively, of either 2.5:1 or 5:1 (corresponding to 130:1 and 260:1 molar ratio, respectively). Mixtures containing C127E media fractions showed partial clearing after 1 h incubation, which was followed by a complete clearing of the mixture containing DMPC–apoE at 2.5:1 ratio by 16 h. In contrast, mixtures containing C127 media fractions did not show any clearing regardless of the incubation period. EM analysis showed that the mixtures containing the C127E media fractions, both at 2.5:1 and 5:1 ratios, contained discoidal particles that stacked on edge and formed rouleaux (**Fig. 8**). The discs had a mean diameter of 13.1 ± 1.8 at 2.5:1 and 14.3 ± 1.9 at 5:1 DMPC–apoE, respectively, with a mean stacking period of 6.1 ± 0.2 nm determined for both mixtures. There was no evidence for the presence of small discoidal particles in mixtures containing DMPC alone or C127 media fractions plus DMPC.

DISCUSSION

The present study demonstrates that expression of human apoE by mouse C127 cells has two major effects on these cells: one is the enhanced secretion of lipids mostly in the form of vesicles, and the other is the formation of nascent lipoprotein-like particles of discoidal shape. The data clearly suggest that the enhanced secretion of lipids was specifically associated with apoE expression since neither mutant apoE nor apoA-I gene expression by these cells resulted in a significant increase in the release of lipids (Fig. 7A). However expression of all three apolipoprotein genes by C127 cells resulted in a minor but significant increase in the synthesis levels as well as the intracellular relative abundance of TAG (Fig. 7B and Table 2, respectively). The significance of this event is not clear at the present time.

Despite the significant increase in the release of lipids by C127E cells, only about 20% of the total secreted apoE is lipid-associated. This could suggest that the amount and the type of lipids in these cells is limiting. However, only 40–50% of the lipids were associated with apoE thus indicating that the enhanced release of lipids could only partly result from a direct association of the expressed apoE with lipids to form lipoprotein-like particles. We have shown that the majority of the particles released by C127E cells (>80%) were vesicles that clearly do not rep-

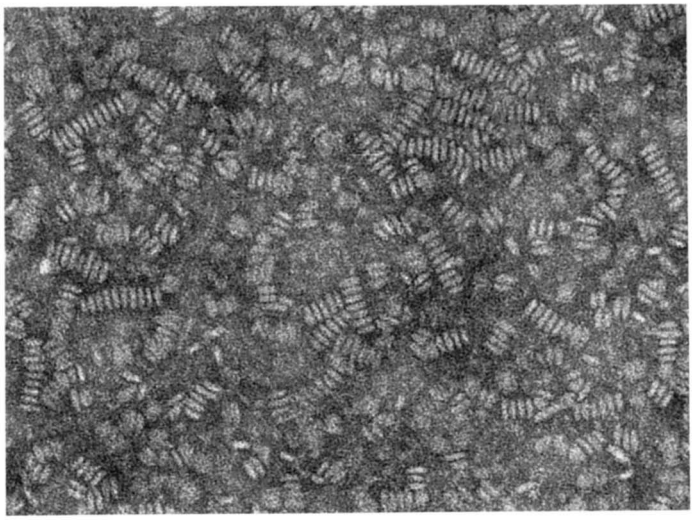

Fig. 8. Electron micrograph of complexes formed from DMPC multilamellar vesicles and lipid-poor apoE. C127E cells were incubated and processed as described in Fig. 5. The d > 1.21 g/ml fraction was collected, dialyzed against PBS, pH 7.4, and added to DMPC multilamellar vesicles (at a 2.5:1 or 5:1 weight ratio of lipid to apoE, respectively), mixed gently, flushed with N_2, and incubated at 24°C for up to 16 h. Samples were mounted onto glow-discharged carbon-coated copper grids using the "Drop method" as described under Materials and Methods. The magnification factor is 186,600.

resent lipoprotein-like particles (24). Nevertheless, about 18% of the particles, 5% circular and 13% discoidal, appeared similar to nascent HDL (44). The discs had a mean diameter of 16.6 ± 4.6 nm which is within the range reported by others for discoidal HDL recovered from acetylated LDL-loaded macrophages (45), HepG2 cells (46), Caco-2 cells (47), rat liver perfusate (13, 44), and apoE-HDL recovered from plasma of lecithin:cholesterol acyl transferase (LCAT)-deficient patients (48). We have calculated that the maximal amount of apoE associated with discs was about 1 μg/mg cell protein per 40-h incubation period. This amount is comparable to that secreted by HepG2 cells (which secrete ~0.54 μg apoE/mg cell protein per 24-h incubation associated with lipids) (46). Thus it appears that: *a*) the properties of an amphiphilic protein such as apoE are the major determinants for the formation of nascent discoidal HDL-like particles, and *b*) the mechanism for HDL formation may not be unique for liver or intestinal cells. The finding that only a small fraction (about 20%) of the total apoE secreted by C127E cells was lipid-associated can be attributed to the lipid moiety, as we have shown that the secreted lipid-poor apoE readily associated with DMPC multilamellar vesicles and formed discs (Fig. 8). The discs appeared very similar in size to the discs formed from mature delipidated human apoE3 and DMPC at the same apoE-DMPC ratio (data not shown) and had a mean diameter of 13.1 ± 1.8 nm. These findings clearly indicate that lipid-poor apoE has the conformation required for disc formation. However, the lipids are packed in vesicular particles that are presumably saturated with apoE (and other proteins). This leaves some 80% of secreted apoE in a lipid-poor form which can interact with exogenously added DMPC. The ability of the lipid-poor apoE present in C127E media to associate with lipoprotein surfaces such as VLDL, LDL, and HDL has been demonstrated by Hussain et al. (30).

As discussed earlier, the majority of the particles released by C127E cells were vesicles. Previous reports have shown the presence of vesicular particles in other biological systems; Hughes et al. (47) have observed the presence of membranous (vesicular) particles in the HDL fraction of the culture media of Caco-2 cells, which appeared in the background of discoidal and small circular particles. These particles were not characterized further. Vesicular, abnormal lipoproteins designated lipoprotein X (LP-X) (49, 50) have been observed in plasma obtained from cholestatic patients. The hepatic origin of these particles has been indicated using the rat liver perfusion system (51). However, unlike the particles present in C127E media, LP-X is recovered from the low density fraction and appears to represent unilamellar vesicles with low protein content.

The release of vesicles has also been observed in cells that are not involved in lipoprotein production, such as murine melanoma cells (52) and blood cells such as lymphocytes (53) and platelets (54, 55). The latter release vesicular particles of variable size to the surrounding environment in response to various stimuli (54). One possible mechanism proposed to explain release of vesicular particles by cells in general is a phenomenon that occurs widely in nature whereby cell surface membranes are shed. Shedding of plasma membrane fragments is an active process that plays an important role in various biological events (for review see ref. 56). Several lines of evidence appear to support the possibility of shedding by C127E and control C127 cells. First, we have shown that the secretion of lipids by these cells is inhibited under conditions where protein synthesis was inhibited by cycloheximide, and secretion of both proteins and lipids was abolished in the presence of colchicine. This indicates that the release of lipids and proteins is an active process requiring functional transport mechanisms presumably involving vesicular movement along the microtubules (57). Second, the parent C127 cells which have been derived from murine mammary carcinoma (31) appear to secrete small amounts of vesicles. This indicates that shedding in these cells occurs regardless of apoE expression and is consistent with the observation that release/shedding of vesicular plasma membrane fragments is a general event manifested by neoplastic cells (56).

The detailed mechanisms involved both in the increased release of lipids by C127E and in the formation of nascent discoidal lipoprotein-like particles are not fully characterized. However, the data reported here clearly suggest that at least some of the processes may occur extracellularly, as shown by the finding that added extracellular apoE (at concentrations comparable to those released by C127E cells) both induced increased lipid release by the parent C127 cells and became associated with some of the released lipids (Table 3). The fact that apoA-I expressed by C127 cells (C127A-I) did not display the same effects as those exerted by apoE further supports the unique potential of apoE to mediate certain cellular events that may not necessarily relate to lipoprotein metabolism. In this regard, possible involvement of apoE in lymphocyte proliferation has been suggested (58).

In summary, our findings suggest that the primary sequence of apoE has the potential to dictate, to a limited extent, its association with surface lipids to form lipoprotein-like particles of discoidal morphology. This assembly could occur either intra- or extracellularly. If the latter takes place, lipid-free apoE can also associate with vesicular plasma membrane fragments shed from C127 cells. This phenomenon of shedding appears to be greatly enhanced by the expression of apoE but not by the expression of either a mutant apoE or normal apoA-I genes.

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