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A plasma lipoprotein containing only apolipoprotein E and with γ mobility on electrophoresis releases cholesterol from cells

(atherosclerosis/cholesterol efflux/high density lipoprotein subclasses/reverse cholesterol transport)

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ABSTRACT **Previous studies have identified lipid-poor high density lipoproteins with electrophoretic pre-β-mobility as the initial acceptors of cell-derived cholesterol in human plasma. These lipoproteins contain apolipoprotein A-I (apoA-I) as their sole apolipoprotein. In the present study, incubation of human plasma with [^{3}H]cholesterol-laden skin fibroblasts has led to the identification of another lipoprotein that serves as a potent initial acceptor of cell-derived cholesterol. This lipoprotein, which we term γ-LpE, exhibits γ mobility on agarose gel electrophoresis. As determined by nondenaturing PAGE and by electron microscopy, the size of the spherical particle ranges between 12 and 16 nm. SDS/PAGE and subsequent immunoblotting identified apoE as its sole apolipoprotein. Plasma from normal and apoA-I-deficient mice, but not from apoE-deficient mice, released [^{3}H]cholesterol from fibroblasts into a γ-migrating lipoprotein. Cell culture media from hepatoma cells or mouse peritoneal macrophages, both of which contain apoE of cellular origin, also promoted efflux of [^{3}H]cholesterol from fibroblasts into a γ-migrating fraction. This was not observed with cell culture medium from fibroblasts alone. In conclusion, our results strongly indicate the presence in human plasma of a lipoprotein containing only apoE, γ-LpE, which is secreted by peripheral cells and is a potent acceptor of cell-derived cholesterol.**

Several epidemiological and clinical studies have revealed an inverse correlation between the plasma concentration of high density lipoprotein (HDL) cholesterol and the risk of myocardial infarction (reviewed in ref. 1). The putative ability of HDL to protect the vessel wall from atherosclerosis has usually been explained by the reverse cholesterol transport model in which HDL mediates the flux of excess cholesterol from peripheral cells to the liver (reviewed in refs. 2–4). In the extracellular compartment, this process is initiated by the uptake of cholesterol from cell membranes by a subgroup of small, discoidal, lipid-poor HDL containing only apolipoprotein A-I (apoA-I) and having pre-β mobility upon electrophoresis (5). We previously reported that cell-derived cholesterol is rapidly transferred from pre-β_1-HDL to other lipoproteins in the order pre-β_2-HDL → pre-β_3-HDL → α-HDL → low density lipoprotein (LDL) (6). The enzyme lecithin–cholesterol acyltransferase directly esterifies a minor proportion of cell-derived cholesterol during its passage through pre-β_3-HDL; most cholesterol, however, is esterified in α-HDL after it has recycled from LDL following transitory storage with the latter lipoproteins (6). According to this scheme, apoA-I is the central protein in the removal of cholesterol from nonhepatic cells. Deficiency or severe reduction of apoA-I should therefore result in HDL deficiency and increased coronary risk (1–4). However, we and others have identified the molecular defects in several families with apoA-I deficiency and familial lecithin–cholesterol acyltransferase deficiency in which, despite HDL deficiency in the homozygous patients and one-half normal HDL-cholesterol levels in heterozygotes, neither patients nor relatives were at increased coronary risk (7–14). This paradox raises the question as to whether proteins other than apoA-I might be important for the removal of cellular cholesterol. During the search for such proteins, we have now identified a previously unknown lipid-poor lipoprotein in human plasma that serves as a potent initial acceptor of cell-derived cholesterol. In contrast to pre-β_1-HDL, this lipoprotein contains only apoE and exhibits γ mobility upon electrophoresis. We have therefore termed it γ-LpE.

MATERIALS AND METHODS

Plasma Samples. Human plasma samples were obtained from three normolipidemic probands after overnight fasting. Streptokinase was used as the anticoagulant at a final concentration of 150 units/ml of blood. Mouse plasma was obtained from normal, apoA-I-deficient, and apoE-deficient mice by retro-orbital puncture and was anticoagulated by the use of streptokinase at a final concentration of 1000 units/ml. The apoA-I and apoE-deficient mice, described previously (15, 16), were generated by gene targeting in embryonic stem cells. The plasma concentration of unesterified cholesterol (UC) in the plasma samples from normal, apoA-I-deficient, and apoE-deficient mice amounted to 14.6 ± 0.8, 9.6 ± 2.6, and 125.5 ± 4.5 mg/dl, respectively.

Cell Culture. Normal human skin fibroblasts were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum in dishes 3.5-cm in diameter as described (17). At the state of near confluence, cells in some dishes were labeled in the presence of fetal calf serum with 0.5 or 0.1 mCi (1 Ci = 37 GBq) of [1,2-^{3}H]cholesterol ([^{3}H]UC; New England Nuclear; 51.7 Ci/mmol) for 72 hr at 37°C. The final specific radioactivity in the washed cells was 2.7 ± 0.8 × 10^8 and 0.6 ± 0.2 × 10^8 cpm/mg of cell protein (mean ± SD), respectively. Peritoneal macrophages were collected from BALB/c mice and cultured in DMEM as described (18). Macrophage-conditioned medium was obtained by loading macrophages with acetyl-LDL (100 μg of protein) for 24 hr followed by incubation for 24 hr with serum-free DMEM. Similarly, HepG2 cell-conditioned medium or fibroblast-conditioned medium was produced by incubating these cells with serum free-medium for 24 hr.

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Abbreviations: apoA, apoB, and apoE, apolipoproteins A, B, and E; LpA and LpE, lipoproteins A and E; HDL, high density lipoprotein; LDL, low density lipoprotein; UC, unesterified cholesterol.

Pulse–Chase Incubations. After being washed six times with phosphate-buffered saline (pH 7.4), radiolabeled cells were incubated with 1 ml of plasma, conditioned cell culture media, or purified γ-LpE (10 μg/ml in DMEM) for 1 min. After a 1-min pulse incubation, plasma samples were removed and either immediately used for agarose gel electrophoresis (see below) or directly transferred to another dish with nonlabeled fibroblasts for another 1-min incubation (chase). All experiments were performed in triplicate.

Electrophoresis. After pulse or chase incubations, plasma samples were sometimes depleted of apoB-containing lipoproteins by incubation with phosphotungstic acid/$MgCl_2$. Aliquots of 20 μl of complete plasma or apoB-free plasma were subjected to electrophoresis in 0.75% agarose gels at 4°C for 2 hr at 40 mA and 300 V using a 50 mM merbital buffer [44.3 g of Tris/19.2 g of Merbital (Serva, Heidelberg, F.R.G.)/0.5 g of calcium lactate/1 g of NaN_3/1000 ml of distilled water, pH 8.6]. Under these conditions, albumin, which was made visible in the unstained gel by adding bromophenol blue to the buffer, had migrated 7 cm. Every lane of the gel was cut into segments of 0.5 cm in length. Lipids were extracted from these slices by incubation with chloroform/methanol (2:1, vol/vol) to determine radioactivity. Alternatively, lipoproteins in the agarose gel were stained with fat red 7B or electroblotted onto nitrocellulose membranes for immunodetection with goat antisera against apoA-I, -A-II, -B, and -E (all from Boehringer Mannheim) and against apoA-IV and apoC (raised in rabbits), biotinylated antibodies against IgG from goat and rabbit, respectively, and streptavidin horseradish peroxidase (Amersham). Lipoproteins migrating within the first 1 cm slice from the starting point were separated in a nondenaturing 2–21% polyacrylamide gradient gel and then electroblotted to nitrocellulose membrane for identification by polyclonal or monoclonal anti-apoE antisera (Boehringer Mannheim).

Isolation of γ-LpE. To characterize γ-LpE, 40 ml of plasma was separated in several agarose gels. From every gel, a 1-cm strip containing the proteins with γ mobility was removed. Proteins from several strips were eluted in phosphate-buffered saline (pH 7.4) and run over an immunoaffinity chromatography column in which purified IgG from anti-apoE antiserum (Boehringer Mannheim) was conjugated to CNBr-activated Sepharose CL-4B (Pharmacia). The bound fraction was eluted with 0.2 M glycine/HCl, pH 3.0. After extensive dialysis with 5 mM ammonium hydrogen carbonate, the eluted lipoproteins were either stained directly for electron microscopy or lyophilized and stored at −20°C.

Analysis of the Protein and Lipid Composition. Proteins from γ-LpE were analyzed by SDS/PAGE (19). The lipids in γ-LpE were quantified using enzymatic photometric tests (i.e., cholesterol and triglyceride with commercially available enzymatic photometric tests from Boehringer Mannheim) and phosphatidylcholine and sphingomyelin as described (20, 21). Total protein concentrations were measured according to Lowry's method (22), using bovine serum albumin as the standard.

RESULTS

Uptake of Cell-Derived Cholesterol by a γ-Migrating Lipoprotein in Human Plasma. Fig. 1 shows the distribution of cell-derived radiolabeled cholesterol throughout human plasma lipoproteins, which were separated by agarose gel electrophoresis. After a 1-min pulse incubation of plasma with radiolabeled fibroblasts, the percentage of [^{3}H]UC in β-, pre-β-, and α-lipoproteins (Lp) amounted to 10% ± 2%, 24% ± 4%, and 34% ± 3%, respectively. The remaining 32% ± 5% was unexpectedly found to be associated with proteins of γ mobility. After a 1-min chase incubation with nonlabeled cells, the radioactivity in this γ fraction decreased from 32% ± 5% to 13% ± 3%, whereas [^{3}H]UC increased in β-Lp to 20% ± 4% and in α-Lp to 50% ± 6%, respectively. Furthermore, fibroblasts labeled with [^{3}H]UC were incubated with plasma that had first been depleted of apoB-containing lipoproteins by phosphotungstic acid/$MgCl_2$ precipitation. Under these conditions, a 1-min pulse incubation still led to the accumulation of radioactivity in the γ fraction. In 1-min chase incubation experiments, the radiolabel consistently disappeared from this γ fraction and appeared in α-Lp. These results suggest the existence of a previously undetected lipoprotein with γ mobility upon agarose gel electrophoresis, which participates in the very early uptake of cell-derived cholesterol by plasma.

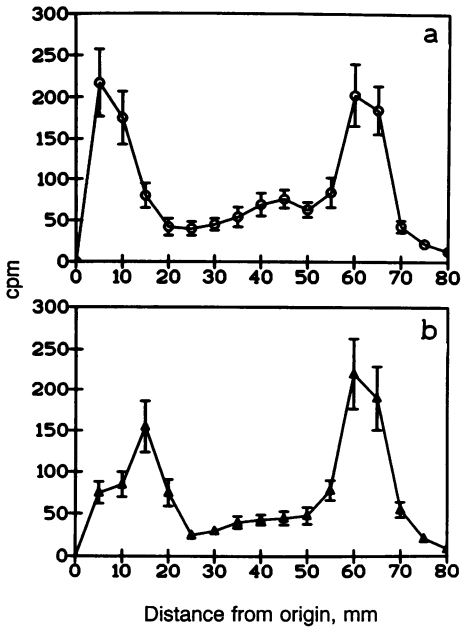

FIG. 1. Distribution of cell-derived cholesterol in various lipoproteins separated by agarose gel electrophoresis. After a 1-min pulse incubation with radiolabeled fibroblasts (*a*) or a further 1-min chase incubation with nonlabeled cells (*b*), 20-μl aliquots of plasma were subjected to electrophoresis in 0.75% agarose gels. The migration distances for lipoprotein standards were 0–10, 11–15, 16–45, and 50–65 mm for γ-, β-, pre-β-, and α-Lp, respectively. After electrophoresis, each lane of the gel was cut into 5-mm-long segments. The lipids in each slice were extracted with chloroform/methanol (2:1), and their radioactivity was determined by liquid scintillation spectrometry. The values presented are the means ± SD of triplicate experiments.

The protein moiety of this lipoprotein was identified using anti-human apolipoprotein antibodies after electroblotting of the agarose gels onto nitrocellulose membranes. Fig. 2*a* presents anti-apoA-I and anti-apoE immunoblots. As expected, most of the anti-apoA-I immunoreactivity was associated with α-Lp, and only a small proportion was associated with pre-β-Lp. Anti-apoE-immunoreactive material was found not only in the α- and pre-β fractions, as expected, but also alone in the γ fraction. Antisera to apoA-II, -A-IV, -B, and -Cs failed to detect any proteins in the γ fraction (data not shown). To rule out cross-reactivity of human gamma globulins with the second biotinylated antibody used for immunodetection, plasma was depleted of IgG by the use of protein G affinity chromatography before being separated by agarose gel electrophoresis. Under these conditions, anti-apoE-immunoreactive material was also detected in a fraction with electrophoretic γ mobility (data not shown; see Fig. 4).

Characterization of γ-LpE. γ-LpE was purified to homogeneity from human plasma by agarose gel electrophoresis and subsequent anti-apoE affinity chromatography. After

isolation γ-LpE still exhibited γ mobility upon agarose gel electrophoresis (Fig. 2*a*). On nondenaturing polyacrylamide gel electrophoresis, γ-LpE presented itself as a disperse band with an apparent particle size of 14 ± 3 nm (data not shown). On electron microscopy, this lipoprotein appeared as a homogenous spherical particle with a diameter of 12–16 nm (Fig. 2*b*). Under both reducing and nonreducing conditions, SDS/PAGE of γ-LpE revealed the presence of a single protein with an apparent molecular mass of 68 kDa that reacted with both monoclonal and polyclonal antibodies against apoE (Fig. 2*c*). The molecular mass is twice that expected for monomeric apoE. To rule out self-aggregation of isolated apoE as the basis for this phenomenon, two-dimensional electrophoresis in the order agarose gel electrophoresis → SDS/PAGE was performed. Subsequent anti-apoE immunoblotting revealed the presence in the γ fraction of an anti-apoE-immunoreactive protein with a molecular mass of 34 kDa, which corresponds to that of monomeric apoE (Fig. 2*c*). Analysis of the lipid composition revealed that γ-LpE consists of 75% ± 4% protein, 5% ± 1% UC, 7% ± 2% phosphatidylcholine, and 13% ± 4% sphingomyelin.

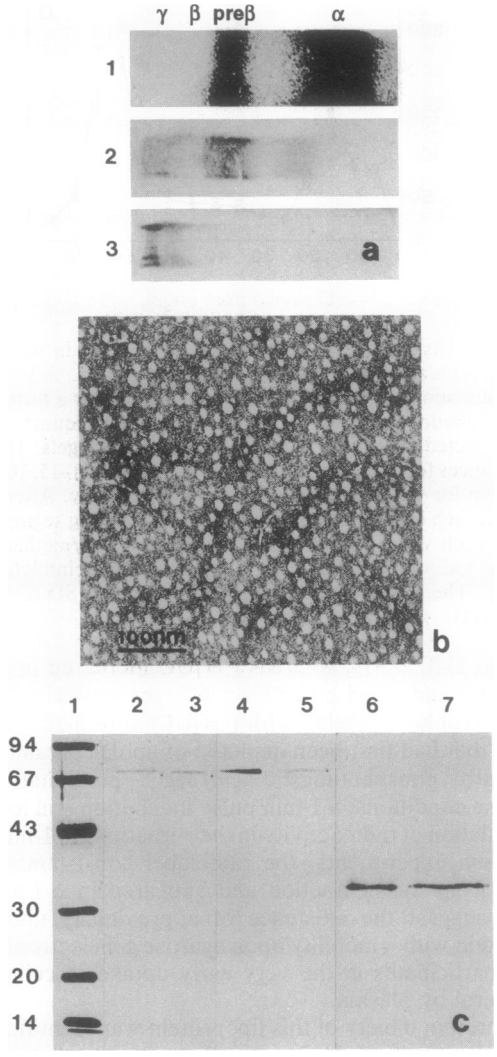

FIG. 2. Characterization of γ-LpE. (*a*) Agarose gel electrophoresis and immunoblotting of apoA-I- or apoE-containing lipoproteins in human plasma. Lane 1, anti-apoA-I immunoblot; lane 2, anti-apoE immunoblot; lane 3, anti-apoE immunoblot of purified γ-LpE. (*b*) Electron micrograph of purified γ-LpE. (*c*) SDS/PAGE of γ-LpE. Lane 1, low molecular mass standards. Lanes 2–5, SDS/PAGE of γ-LpE isolated from plasma by agarose gel electrophoresis and subsequent anti-apoE immunoaffinity chromatography. Lanes 2 and 3, gel stained with Coomassie blue; lanes 4 and 5, anti-apoE immunoblots. Under these conditions apoE presents itself as a protein of 68 kDa, which is twice as high as expected. Lanes 6 and 7, agarose gel strips containing proteins with electrophoretic γ mobility were subjected to SDS/PAGE for subsequent anti-apoE immunoblotting without prior elution of proteins. Under these conditions, apoE is presented with its expected molecular mass of 34 kDa. In lanes 2, 4, and 6, proteins were reduced by incubation of proteins or gel strips with mercaptoethanol. In lanes 3, 5, and 7, SDS/PAGE was performed under nonreducing conditions. In lanes 3–7, immunodetection was performed with biotinylated sheep anti-human apoE antibodies and streptavidin-horseradish peroxidase.

Effect of apoA-I Deficiency and apoE Deficiency on the Uptake of Cell-Derived [^{3}H]UC by γ-LpE. To test the importance of apoE for the uptake of cell-derived cholesterol into a γ-migrating lipoprotein, we repeated the pulse incubations with [^{3}H]cholesterol-loaded fibroblasts using plasma from normal, apoA-I-deficient, and apoE-deficient mice (Fig. 3). Like human plasma, normal murine plasma takes up [^{3}H]UC from fibroblasts into lipoproteins with γ (35% ± 6%), pre-β (7% ± 1%), and α-mobility (53% ± 6%) (Fig. 3*a*). When its low cholesterol concentration is taken into account, murine apoA-I-deficient plasma removed [^{3}H]UC from cells as efficiently as normal plasma (1820 ± 250 vs. 1882 ± 314 cpm/μg of UC); the radioactivity was regularly distributed throughout γ-, pre-β-, and α-Lp (Fig. 3*b*). After pulse incubation of [^{3}H]UC-loaded fibroblasts with apoE-deficient plasma, the specific radioactivity in the medium amounted to only 5% of

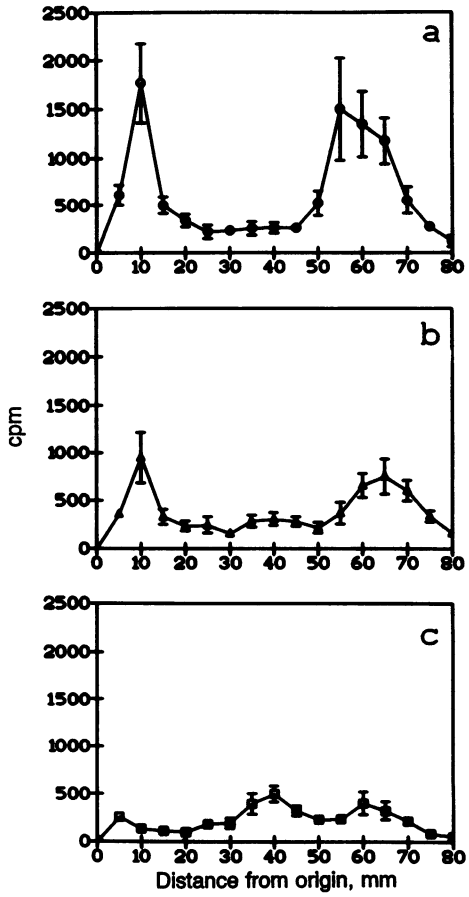

FIG. 3. Distribution of cell-derived [^{3}H]UC in various lipoproteins of plasma from normal, apoA-I-deficient, and apoE-deficient mice. Normal (*a*), apoA-I-deficient (*b*), and apoE-deficient (*c*) plasmas were pulse incubated for 1 min with [^{3}H]UC-loaded human skin fibroblasts. Aliquots of 20 μl of plasma were then subjected to electrophoresis in 0.75% agarose gels. After electrophoresis, each lane of the gel was cut into 5-mm-long segments. The lipids in each slice were extracted with chloroform/methanol (2:1), and their radioactivity was determined by liquid scintillation spectrometry. The values given are the means ± SD of triplicate experiments.

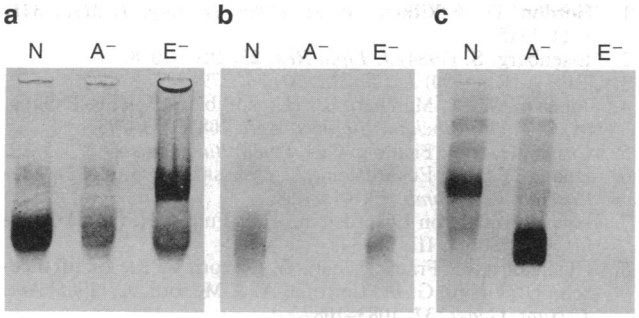

FIG. 4. Agarose gel electrophoresis of lipoproteins from plasmas of normal, apoA-I-deficient, and apoE-deficient mice. To prevent cross-reactions with the second antibody during immunodetection, plasma samples from normal (N), apoA-I-deficient (A^-), and apoE-deficient (E^-) mice were depleted of IgG by protein G immunoaffinity chromatography. Plasma samples were then separated by agarose gel electrophoresis. The gels were either stained with fat red 7B (*a*) or electroblotted to nitrocellulose membranes for immunodetection of apoA-I (*b*) and apoE (*c*). Note the absence of anti-apoE-immunoreactive material in the γ-fraction of apoE-deficient plasma (*c*; E^-).

that obtained after incubation with normal plasma (90 ± 9 cpm/μg of UC). No radioactivity was associated with proteins of γ mobility (Fig. 3*c*).

Fig. 4 demonstrates the electrophoretic separation of murine lipoproteins in normal, apoA-I-deficient, and apoE-deficient plasma that was initially depleted of IgG by protein G affinity chromatography. Lipid staining of the gel revealed similar patterns in normal and apoA-I-deficient mice, but an abnormal pattern with a broad β band in apoE-deficient plasma was observed (Fig. 4*a*). Western blotting revealed anti-apoA-I-immunoreactive material in pre-β- and α-Lp from normal and apoE-deficient plasma (Fig. 4*b*) and anti-apoE-immunoreactive material in γ-, pre-β-, and α-Lp from normal and apoA-I-deficient plasma (Fig. 4*c*). The plasma of apoE-deficient mice contained no anti-apoE or anti-apoA-I-immunoreactive material associated with γ-migrating proteins. These data indicate that the absence of apoE from plasma prevents the formation of γ-LpE and the uptake of cell-derived UC into this lipoprotein.

The Origin of γ-LpE. To exclude the possibility that fibroblasts themselves produce γ-LpE, cell culture medium derived from nonlabeled fibroblasts (fibroblast-conditioned medium) was pulse incubated with radiolabeled fibroblasts and subsequently separated by agarose gel electrophoresis. After incubation for up to 20 hr, no significant radioactivity was detectable in the γ fraction of an agarose gel (Fig. 5*a*). However, addition of 10 μg of γ-LpE either to the fibroblast-conditioned medium or directly to the radiolabeled fibroblasts resulted in significant accumulation of [^{3}H]UC in a distinct γ fraction after a 1-min incubation (Fig. 5*b*). We also tested whether apoE-synthesizing cells produce γ-LpE. Medium from either cultured mouse peritoneal macrophages (Fig. 5*c*) or HepG2 cells (Fig. 5*d*) was pulse incubated for 1 min with radiolabeled fibroblasts. Considerable amounts of radiolabeled cholesterol were found associated with γ-migrating lipoproteins, suggesting that these cells indeed produce γ-LpE.

DISCUSSION

In normal human plasma, apoE is a structural component of chylomicrons, very low density lipoproteins, intermediate density lipoproteins, and HDL_1 among which it is exchanged in response to metabolic changes of plasma lipids (23). Furthermore, isolation of lipoproteins by various techniques sometimes leads to the dissociation of apoE. For example, ultracentrifugation of plasma at $d < 1.21$ g/ml leads to the occurrence of some apoE unassociated with apoA-I or apoB in the lipoprotein-free infranatant (24). By contrast, after anti-apoE immunoaffinity chromatography of plasma, all apoE was found associated with apoA-I or apoB. Therefore, it was concluded that apoE is present in normolipidemic plasma only as a component of apoA-I- and apoB-containing lipoproteins. However, plasma of patients with familial lecithin–cholesterol acyltransferase deficiency contains a discoidal, apoE-containing lipoprotein that promotes cholesterol efflux from fibroblasts (17, 25, 26). This study describes the presence in normal human and murine plasma of a

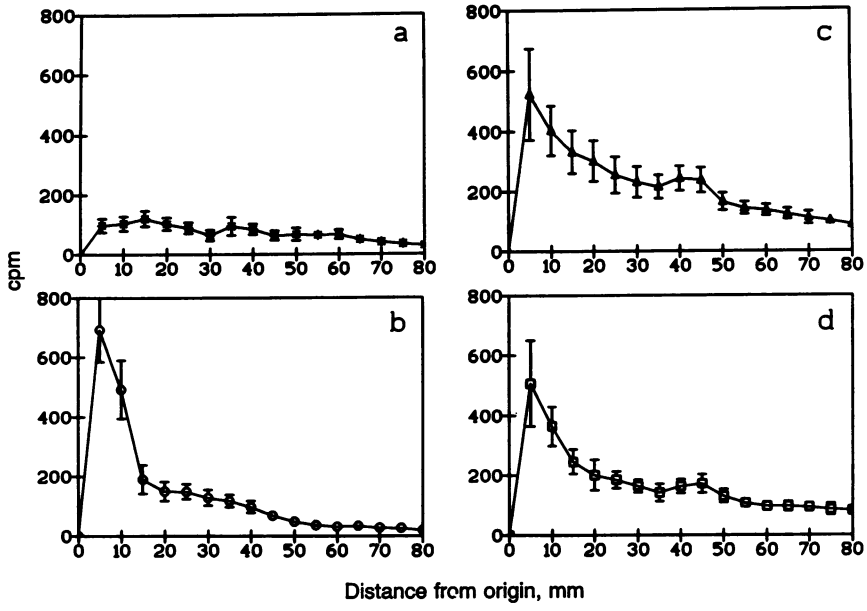

FIG. 5. Electrophoretic distribution of cell-derived [^{3}H]UC in conditioned media from various cells. One milliliter of fibroblast-conditioned medium (*a*), fibroblast-conditioned medium plus 10 μg of γ-LpE (*b*), macrophage-conditioned medium (*c*), or HepG2 cell-conditioned medium (*d*) was incubated with radiolabeled fibroblasts for 1 min at 37°C. Other experimental conditions correspond to those described in Fig. 1. Note that medium from fibroblasts releases [^{3}H]UC into a γ-migrating lipoprotein only if supplemented with γ-LpE (*b*). The values given are the means ± SD of triplicate experiments.

lipoprotein, γ-LpE, that also contains apoE as the only protein constituent and sphingomyelin as the major lipid component. It is a spherical particle with a diameter of 12–16 nm and exhibits γ mobility on agarose gel electrophoresis. It is also a potent acceptor of cell-derived cholesterol.

In its ability to take up cell-derived UC and to subsequently transfer it to α-HDL, γ-LpE functionally resembles pre-β_1-HDL (pre-β_1-LpA-I), a discoidal particle that contains apoA-I as the only protein constituent and that has so far been considered to be the only particle involved in the initial uptake of cell-derived cholesterol (5). In our studies, γ-LpE contained in normal human and murine plasma samples took up more cell-derived [^{3}H]UC than pre-β_1-LpA-I. To further substantiate this important role of γ-LpE in the uptake of cellular cholesterol, [^{3}H]UC-labeled human fibroblasts were incubated with plasma from both apoA-I- and apoE-deficient mice. In these experiments, plasma from apoA-I-deficient mice regularly released cell-derived [^{3}H]cholesterol into the γ-LpE fraction. By contrast, apoE-deficient murine plasma failed to take up cell-derived [^{3}H]UC into this lipoprotein with γ mobility. Moreover, the specific radioactivity released from the cells by incubation with apoE-deficient plasma amounted to only 5% of that obtained after incubation with normal plasma, whereas apoA-I-deficient plasma released regular amounts of specific radioactivity. These observations strongly suggest that γ-LpE is very important, at least in murine plasma, in promoting cholesterol efflux from cells. Furthermore, in contrast to apoA-I, which is synthesized only by hepatocytes and enterocytes, apoE is secreted by a broad variety of cells including macrophages (23, 27–29). This ubiquity of apoE synthesis further supports an important role of γ-LpE in the removal of cholesterol from cells, and possibly, thereby in the protection of the vessel wall from atherosclerosis. Differentiated macrophages, which predominate in the atherosclerotic plaque, increase their synthesis of apoE when loaded with cholesterol (18, 28). In the present study, we demonstrated that medium preconditioned by incubation with cholesterol-loaded macrophages not only releases [^{3}H]UC from fibroblasts into a γ-migrating lipoprotein but also releases [^{3}H]UC from mouse peritoneal macrophages (data not shown). This indicates, as originally suggested by Basu *et al.* (18), that macrophages may deplete themselves of excess cholesterol by secretion of apoE. This conclusion gains further support from the *in vivo* observations that infusion of apoE leads to the regression of atherosclerosis in LDL receptor-deficient Watanabe heritable hyperlipidemic rabbits (30) and that apoE-deficient mice but not apoA-I-deficient mice suffer from atherosclerosis (31–33). ApoE deficiency in mice (31, 32) and humans (34–36) might thus cause atherosclerosis not only by impairment of remnant catabolism and hypercholesterolemia but also by impairing the efflux of cholesterol from cells.

In conclusion, we have identified an apoE-containing lipoprotein, γ-LpE, that takes part in the very early stages of the uptake and transfer of cell-derived cholesterol by human plasma. Since apoE is secreted by many cells including arterial wall macrophages, the presence of γ-LpE in plasma may indicate a general mechanism by which cells rid themselves of excess cholesterol.

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