Association of human apolipoprotein E with lipoproteins secreted by transfected McA RH7777 cells

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Abstract To examine the association of apolipoprotein (apo) E with nascent hepatic lipoproteins we have prepared stable transfectants of the rat hepatoma cell line McA RH7777 expressing the human apoE3 cDNA. When the nascent lipoproteins secreted from control cells were separated on fast protein liquid chromatography (FPLC) columns, rat apoE was detected in the very low density (VLDL) and high density lipoprotein (HDL) fractions, while rat apoA-I was found in the HDL and lipoprotein free fractions. Human apoE was also associated with the VLDL and HDL particles secreted from the transfected McA RH7777 cells. Expression of human apoE resulted in a significant decrease in the amount of rat apoA-I associated with the lipoprotein particles. Rat apoE was also displaced, but to a lesser extent. Infection of McA RH7777 cells at different multiplicities of infection with recombinant adenoviral vector containing the human apoE cDNA indicated that rat apoA-I was decreased in the HDL fractions at lower levels of expression of human apoE than was rat apoE. The HDL particles were further examined by immunoblotting of non-denaturing gradient gels and by non-denaturing immunoprecipitation. The results indicate that the high density lipoprotein (HDL) particles are heterogeneous in size and apolipoprotein composition with the majority of the rat and human apolipoproteins being located on different particles. These results suggest that the profile and concentration of HDL apolipoproteins produced in hepatocytes influences the assembly of the various subsets of secreted HDL.— Reardon, C. A., L. Blachowicz, K. M. Watson, E. Barr, and G. S. Getz. **Association of human apolipoprotein E with lipoproteins secreted by transfected McA RH7777 cells. *J. Lipid Res.* 1998.** 39: **1372–1381.**

Supplementary key words assembly of HDL • apolipoproteins

Apolipoprotein (apo) E is a component of several classes of lipoproteins, including chylomicron remnants, VLDL and HDL, although apoE is not uniformly distributed on VLDL and HDL particles. As apoE is a ligand for a variety of cell surface receptors (1), its presence on a lipoprotein particle influences the metabolic fate of the host particle. In addition, the composition and size of the lipoprotein particles containing apoE has been shown to differ from apoE-free lipoproteins with the same density. ApoE-containing VLDL have more free cholesterol, cholesteryl esters, and sphingomyelin than apoE-free VLDL (2). HDL containing apoE are generally larger and contain more cholesteryl esters than HDL lacking apoE (3) and they bind with high affinity to the LDL receptor (4). These latter particles are believed to participate in the transport of excess cellular cholesterol in peripheral cells to the liver, especially in mammalian species that lack cholesteryl ester transfer protein.

Support for the importance of apoE in lipoprotein metabolism comes from the occurrence of natural LDL receptor binding mutants of human apoE and studies in mice. Mutations in apoE, especially those near the LDL receptor binding domain, decrease the binding of apoE to the receptor and are associated with plasma lipid and lipoprotein abnormalities (5, 6). On the other hand, overexpression of rat apoE in transgenic mice results in a reduction in plasma triglyceride and cholesterol levels due to an enhanced clearance of VLDL, LDL, and chylomicron remnants (7, 8). HDL levels were not as dramatically affected. ApoE transgenic mice also exhibited a resistance to diet induced hypercholesterolemia (7). In contrast, apoE deficiency in mice generated by homologous recombination results in the accumulation of cholesterol and triglycerides associated with lipoprotein remnants (9, 10). The hypercholesterolemia of apoE-deficient mice can be reversed by transplantation of bone marrow cells from apoE-producing mice (11, 12) and by the injection of recombinant adenovirus expressing human apoE3 (13, 14).

All of the soluble apolipoproteins contain a variable number of 11 or 22 amino acid tandem repeats that are

Abbreviations: apo, apolipoprotein; CE, cholesteryl esters; CMV, cytomegalovirus; DMEM, Dulbecco's modified Eagle's medium; ECL, enhanced chemiluminescence; FC, free cholesterol; HDL, high density lipoprotein; HuE, human apoE; hEs, anti-human apoE specific antibodies; rEs, anti-rat apoE specific antibodies; LDL, low density lipoprotein; LPDS, lipoprotein-deficient serum; Neo, neomycin; PL, phospholipids; TG, triglycerides; TLC, thin-layer chromatography; VLDL, very low density lipoprotein.

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capable of forming amphiphathic α-helical structures of three different types (15). These amphipathic structures are capable of binding to phospholipid vesicles in vitro and are believed to be important for lipoprotein association. We have begun to examine the association of apolipoproteins with nascent hepatic lipoproteins by expressing apoE/apoA-I chimeric apolipoproteins in the rat hepatoma cell line McA RH7777 and have shown that the C-terminal amino acids encoded by the fourth exon of apoE are important for its association with VLDL and HDL particles (16). In vitro studies examining the association of apoE truncated mutants with mature plasma lipoproteins suggest that residues 260–272 are important for the association of the apolipoproteins with VLDL and HDL (17, 18). In particular, residues 260–266 may contain an important determinant for VLDL binding. However, the association of apoE with different classes of lipoproteins is complex and probably involves interactions between different domains of the protein. The apoE3 isoform of apoE which contains a cysteine at position 112 preferentially associates with HDL, while apoE4 with an arginine at position 112 and a variant of apoE3, in which there is a duplication of amino acids 121–127 (apoE3-Leiden), preferentially associate with VLDL (19, 20). The preferential association of apoE4 with VLDL suggests that the region around residue 112 modifies the lipid/lipoprotein interactions mediated by the last 55 amino acids of the protein. This is likely due to the formation of a salt bridge in apoE4 between Glu^{109} and Arg^{112} that alters the orientation of Arg^{61} thus allowing it to interact with Glu^{255} (21), although how this influences lipoprotein association is not clear.

The McA RH7777 cell line has been shown to secrete VLDL- and HDL-like particles. The density <1.06 g/ml particles secreted by the non-transfected McA RH7777 cells consist of small VLDL and IDL containing primarily apoB-100, apoB-48, and apoE (22). The HDL particles are primarily spherical with only a small proportion of disk-shaped particles and contain apoE and apoA-I. ApoB-48 is also found on particles within the HDL range. In this study we have explored the interactions of apolipoproteins that potentially associate with the nascent HDL secreted by stable transfected McA RH7777 cells in determining the HDL apolipoprotein profile. We asked the following questions. *a*) Are endogenous rat apoE and apoA-I secreted on the same particles or on lipoprotein particles with similar properties but different apolipoprotein composition? *b*) Does overexpression of human apoE influence the lipoprotein association of endogenous rat apoE and apoA-I? *c*) Is the influence of overexpressed apoE dependent on the level of overexpression? Our results suggest that rat apoE, rat apoA-I, and human apoE compete with one another for the pool of secreted phospholipid in assembling the lipoproteins with which they are associated.

EXPERIMENTAL PROCEDURES

Materials

Tran^{35}S-label (specific activity ~1000 Ci/mmol) and [1, 2, 3-^{3}H]glycerol (specific activity 200 mCi/mmol) were purchased from ICN Biomedicals, Inc. and New England Nuclear, respectively. [1α, 2α(n)-^{3}H]cholesterol and ^{125}I-protein A (specific activity > 30 mCi/mg) were purchased from Amersham Corp. High glucose Dulbecco's modified Eagle's medium (DMEM), l-methionine-free DMEM, fetal bovine serum, horse serum, l-glutamine, penicillin-streptomycin, trypsin and ImmunoSelect were supplied by Gibco Life Technology, Inc. Immobilon-P was obtained from Millipore Corp. BioCount scintillation fluid was obtained from Research Products International. Non-denaturing gradient gels were supplied by Dr. David Raintree (San Antonio, TX) and molecular weight standards were obtained from BioRad. Enhanced chemiluminescence (ECL) kit was the product of Amersham Corp. Horseradish peroxidase-coupled anti-rabbit IgG, protease inhibitors, and G418 were supplied by Sigma Chemicals. Centriprep 10 and Centricon 10 columns were obtained from Amicon. Plastic-backed Silica gel 1B2 thin-layer chromatography plates were obtained from Baker. All other reagents were of the highest quality available.

Preparation of expression vectors

The pCMV4 expression vector (23) was kindly supplied by Dr. David Russell, University of Texas. The AatII-HinfI fragment of the apoE cDNA was subcloned into the KpnI/XbaI sites.

McA RH7777 cells

McA RH7777 cells were obtained from American Type Culture Collection, Rockville, MD (ATCC number CRL 1601). The cells were maintained in high glucose (4500 g/liter) DMEM with 10% fetal calf serum, 5% horse serum, and 1% penicillin/streptomycin. The cells were transfected by the calcium phosphate coprecipitation method with glycerol shock (24) with pSV2-Neo alone or together with the cDNA expression vector. Cells were selected in 400 μg/ml G418 and individual colonies were analyzed for their ability to synthesize human apoE by Western blot analysis using human apoE specific antibodies. The transfected cells were maintained in media containing 200 μg/ml G418 for no more than 3 months, during which time no changes in human apolipoprotein expression levels were observed. To quantitate the relative levels of secretion of the apolipoproteins by the transfected cells, McA RH7777 cells were seeded in 12-well plates at 2.3×10^5 cells per well. After 24 h the media were replaced with methionine-free DMEM, 10% lipoprotein-deficient serum (LPDS), and 100 μCi/ml Tran^{35}S-label and the cells were incubated for 18 h. Equal trichloroacetic acid-precipitable counts of media were immunoprecipitated with specific antibodies and ImmunoSelect and analyzed on 5–22.5% polyacrylamide SDS gels as described (25). After fluorography, the apoE bands were excised from the gels, solubilized in 30% hydrogen peroxide and 69–72% perchloric acid overnight at 60°C, and counted in scintillation fluid. To calculate relative levels of expression of the apolipoproteins, the presence of 10 methionines in rat apoE and 7 methionines in human apoE was taken into account. The apolipoproteins were quantitatively immunoprecipitated as evidenced by the fact that a second immunoprecipitation of the supernatant with the same antibody did not yield additional labeled protein.

LPDS was prepared from heat-inactivated fetal calf serum (56°C, 30 min) by centrifuging twice for 48 h at 55,000 rpm after adjusting the density to 1.25 g/ml with NaBr. The sample was dialyzed against phosphate-buffered saline, pH 7.0, containing 1 mm EDTA and sterilized using a 0.22-μm syringe filter and stored at −20°C. LPDS was used in the media at a final concentration of 10% (2.5 mg protein/ml) or 1% (0.25 mg/ml).

Infection of McA RH7777 cells with recombinant human apoE adenovirus

McA RH7777 cells were seeded at a density of 1.2×10^6 cells in a 25-cm^2 flask. After 24 h, the media were replaced with se-

rum-free high glucose DMEM and 0–20 × 10^6 pfu of recombinant human apoE3 adenovirus was added. The media were removed after 2 h and replaced with high glucose DMEM containing 1% LPDS for 18 h. The media were concentrated and fractionated on FPLC columns as described below. The recombinant adenovirus was prepared by subcloning the BalI-HinfI fragment of the human apoE cDNA into the BamHI site of AdEF1(KN) transfer vector and cotransfecting HEK-293 cells (ATCC number CRL 1573) with the resulting plasmid and Ad5sub360 adenoviral DNA (26).

FPLC columns

McA RH7777 cells were seeded at a density of 4.5 × 10^6 cells per 75-cm^2 flask. After 24 h, the media were replaced with 10 ml of high glucose DMEM containing 1% LPDS for an additional 18 h. Preservatives (1 mm PMSF, 0.2% aprotinin, 0.01% EDTA, 0.002% Na azide, and 1 mm BHT) were added to the media and the media from 2 flasks were concentrated to 500 μl using Centriprep 10 and Centricon 10 columns and applied to tandem Superose 6 columns (Pharmacia). The lipoproteins were eluted with 0.02 m sodium phosphate, pH 7.4, 0.05 m NaCl, 0.03% EDTA and 0.02% sodium azide and 500-μl fractions were collected. Aliquots (50 μl) of each fraction were subjected to electrophoresis on 5–22.5% gradient polyacrylamide SDS gels. The proteins were transferred to Immobilon-P and probed with specific antibodies and 1 μCi ^{125}I-protein A. After exposure to X-ray film, the bands corresponding to the apolipoproteins were excised and counted in a gamma counter. The area under the peaks was quantitated by a computer digitizer (Sigma Scan, Scientific Measurement System, Jandell Scientific) and expressed as a percentage of total areas. Human plasma was fractionated on the Superose 6 columns as described above to determine the elution profile of each of the plasma lipoproteins and lipoprotein free proteins.

NaBr density gradients

McA RH7777 cells were seeded in 25-cm^2 flasks at a density of 1.5 × 10^6 cells per flask. After 24 h, the media were removed and replaced with high glucose DMEM containing 10% LPDS. The media were removed after 18 h and preservatives were added. The lipoproteins in 2 ml of media were separated by centrifugation on 3–20% NaBr gradients in an SW41 rotor for 66 h at 35,000 rpm (27). One-ml fractions were collected, dialyzed against Tris-buffered saline (10 mm Tris, 150 mm NaCl, 0.01% EDTA, 20 mm NaN_3, pH 7.4) and 120 μl of each fraction was subjected to electrophoresis on 5–22.5% gradient polyacrylamide SDS gels, transferred to Immobilon-P, probed with specific antibodies and ^{125}I-protein A, and quantitated as described for the FPLC columns. The density of the individual fractions was determined using a refractometer.

Non-denaturing gradient gels

To determine the size of the secreted HDL particles, 50 and 75-μl aliquots of media or the HDL fractions from the FPLC column or equilibrium density gradients were loaded onto 4–30% non-denaturing gradient gels, subjected to electrophoresis in 90 mm Tris, 80 mm boric acid, 2.5 mm sodium azide, 0.025 mm EDTA, pH 8.4, at 150 volts for 18 h, and transferred to Immobilon-P membrane overnight at 0.36 Amp per gel using 0.025 m Tris, 0.192 m glycine, and 0.02% SDS. After incubation with specific antibodies, the proteins were visualized using ECL detection. Proteins of known radii were included as standards: thyroglobulin, 8.5 nm; ferritin, 6.1 nm; catalase, 4.6 nm; lactate dehydrogenase, 4.1 nm; and albumin, 3.55 nm.

Non-denaturing immunoprecipitations

McA RH7777 cells were seeded in 75-cm^2 flasks at 4.5 × 10^6 cells per flask. After 24 h the media were replaced with methionine-free DMEM, 1% LPDS, and 100 μCi/ml Tran^{35}S-label and the cells were incubated for an additional 18 h. Preservatives were added to the media, the media were concentrated and subjected to separation on FPLC columns as described above. HDL fractions were pooled, adjusted to 0.2 m NaCl, and subjected to non-denaturing immunoprecipitation. The samples were incubated with specific antibodies for 16–18 h at 4°C, followed by ImmunoSelect for 4 h at room temperature, and the precipitate was washed three times with 10 mm HEPES, pH 7.6, with 250 mm NaCl. The washed pellet was dissociated with Laemlli sample buffer and applied to 9–13% polyacrylamide SDS gels. The radioactive proteins were visualized by fluorography and quantitated as described above. To determine whether the HDL particles secreted from the McA-HuE cells contained both rat and human apoE, after the non-denaturing immunoprecipitation using a species-specific antibody (antibody 1), the washed pellet was dissociated in 0.2% SDS/1% Triton X-100/1% deoxycholate, and subjected to denaturing immunoprecipitations using the opposite species specific antibody (antibody 2) as described (25).

Lipid labeling

Two 150-cm^2 flasks were seeded with 9 × 10^6 cells per flask. After 24 h, the media were replaced with 10 ml of high glucose DMEM containing 1% LPDS with 3 μCi/ml [^{3}H]glycerol or 0.5 μCi/ml [^{3}H]cholesterol with 5 μg/ml cholesterol for an additional 18 h. The media were collected and fractionated on FPLC columns as described above and the peak HDL fractions were pooled. The HDL pools were subjected to non-denaturing immunoprecipitation using non-immune serum or antibodies to rat apoA-I, rat apoE, or human apoE as described above. The pooled HDL fractions and the immunoprecipitates were subjected to Bligh-Dyer extraction (28) and the extracted lipids were spotted onto silica gel plates along with lipid standards. The plates were developed in petroleum ether–ethyl ether–acetic acid 75:25:1. Lipid standards were visualized by iodine vapors and the regions corresponding to triglycerides and phospholipids for glycerol-labeled cells and free cholesterol and cholesteryl esters for cholesterol-labeled cells were excised and counted in scintillation fluid. The amount of lipid in the HDL fractions was expressed as cpm of label per mg cell protein. Cell protein was extracted by incubation of the cells with 0.1 N NaOH overnight and quantitated by the method of Lowry et al. using bovine serum albumin as a standard (29).

Antibodies

Antibodies to apoproteins were prepared in New Zealand rabbits using proteins purified from rat and human plasma lipoproteins on SDS-polyacrylamide gels. To prepare species-specific apoE antibodies, anti-human apoE antiserum was passed through a rat HDL-Sepharose column and anti-rat apoE antiserum was passed through a human VLDL-Sepharose column to remove cross-reacting species from the polyclonal antisera. The species-specific nature of the apoE antibodies was examined and validated by immunoprecipitation of ^{35}S-labeled media from HepG2 and McA RH7777 cells (data not shown) and by Western blot analysis of mouse and human lipoproteins and media from McA-Neo cells using ECL detection (**Fig. 1**).

RESULTS

Expression of human apoE in McA RH7777 cells

To examine the association of human apoE with nascent hepatic lipoproteins, the rat hepatoma cell line McA RH7777 was stably transfected with an expression vector

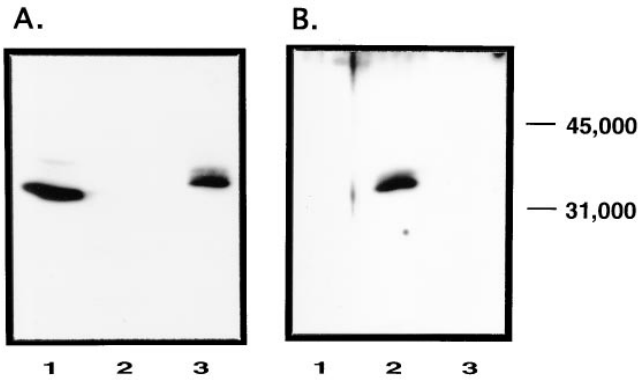

Fig. 1. Species specificity of antibodies to rat and human apoE. Four microliters of mouse plasma (lane 1), 4 μl of human plasma (lane 2), and 12 μl of media from McA-Neo cells (lane 3) were electrophoresed on 5–22.5% acrylamide-SDS gels and transferred to Immobilon-P membrane. The membranes were probed with antiserum against rat (panel A) and human (panel B) apoE that were made species specific by passing the rabbit antiserum through a human VLDL-Sepharose column and a rat HDL-Sepharose column, respectively.

containing the apoE3 cDNA and the cytomegalovirus (CMV) promoter. Individual colonies of cells were examined for the ability to secrete human apoE into the media. We selected for extensive study the colony that expressed the highest level of human apoE, although other colonies showed qualitatively similar results. The relative level of expression of endogenous rat apoE and rat apoA-I and the human apoE secreted from control neo transfected cells (McA-Neo) and the human apoE transfected cells (McA-HuE) was determined by immunoprecipitation of ^{35}S-labeled media proteins using species-specific antibodies (**Table 1**). The amount of rat apoE and rat apoA-I in the media of the human apoE expressing cells is expressed relative to the amount of the apolipoprotein secreted into the media by the McA-Neo cells. The expression of human apoE in the transfected McA-HuE cells had no effect on the secretion of these endogenous apolipoproteins. The amount of human apoE secreted by the McA-HuE cells is 2.6 times that of the endogenous rat apoE. Although most of the results detailed below were obtained with cells transfected with the apoE cDNA driven by the CMV promoter, essentially the same qualitative results were obtained when a vector containing the apoE cDNA driven by the metallothionein promoter (30) was used (data not shown). The level of human apoE secreted from these latter cells was only 26% of the level of rat apoE secreted from the same cells.

TABLE 1. Relative level of apolipoproteins secreted from transfected cells

Cell Line	Rat ApoE	Rat ApoA-I	Human/Rat ApoE
McA-Neo	1.00	1.00	—
McA-HuE	0.96	0.94	2.65

Cells were seeded into 12-well plates, pulsed with Tran^{35}S-label, and immunoprecipitated using species-specific antibodies as described in Experimental Procedures. The radioactive bands corresponding to the apolipoproteins were counted and the relative amounts of rat apoE and rat apoA-I secreted from the human apoE transfected cells are expressed relative to the amount of the protein secreted from McA-Neo control cells. The amount of human apoE secreted is expressed relative to the amount of rat apoE secreted from the same transfected cell.

Association of apolipoproteins with nascent particles secreted from McA RH7777 cells

To examine the distribution of the endogenous rat apolipoproteins and human apoE on the lipoproteins secreted from the McA RH7777 cells, the lipoproteins secreted into LPDS containing media were separated on tandem Superose 6 FPLC columns. In McA-Neo transfected cells (**Fig. 2A**) rat apoA-I was found in the HDL fractions and in the lipoprotein-free fractions. Scanning of the graphs indicated that 20.9 ± 2.5% of the total apoA-I was found in the lipoprotein free fractions. Rat apoE was found primarily on HDL particles with a small amount on the VLDL particles. Very little of this apolipoprotein was found in the lipoprotein-free fractions (5.2% of total rat apoE). Human apoE secreted from the McA-HuE cells (Fig. 2B) was detected in both the VLDL and HDL fractions with very little of the apolipoprotein in the lipoprotein-free fractions. The expression of human apoE in these cells resulted in a 3.3 ± 0.3-fold increase in the amount of rat apoE in the lipoprotein-free fractions. There does not appear to be a significant effect on the amount of rat apoE associated with the VLDL fractions, suggesting that rat and human apoE compete for the HDL surface with the result that more rat apoE is secreted free of lipid. Expression of human apoE had an even more dramatic effect on the distribution of rat apoA-I. The amount of rat apoA-I associated with the HDL particles secreted from the McA-HuE cells was barely detectable and the majority of the recovered rat apoA-I was in the lipoprotein–free fractions.

Similar results were obtained when the association of apolipoproteins with HDL was examined on 3–20% NaBr equilibrium single spin density gradients (data not shown). In McA-Neo transfected cells, rat apoE and rat apoA-I are associated with particles with densities 1.08–1.13 g/ml. Human apoE also associated with HDL particles with density 1.07–1.13 g/ml. Upon isolation of the lipoproteins on density gradients we estimate that the percent of apoA-I in the lipid-poor fractions increased from 34% ± 8.9 (n = 6) of the apoA-I in the d > 1.06 g/ml fractions in McA-Neo transfected cells to 66.5% ± 13.4 (n = 5) in McA-HuE cells.

To evaluate the influence of different levels of human apoE expression on apolipoprotein distribution among lipoprotein classes, McA RH7777 cells were infected at different multiplicities of infection with recombinant adenovirus containing human apoE3. The secreted lipoproteins were examined on FPLC columns for the distribution of rat apoE, rat apoA-I and human apoE (**Fig. 3**). At the lowest level of infection (20×10^4 pfu per dish), the distribution of the rat apolipoproteins was not altered.

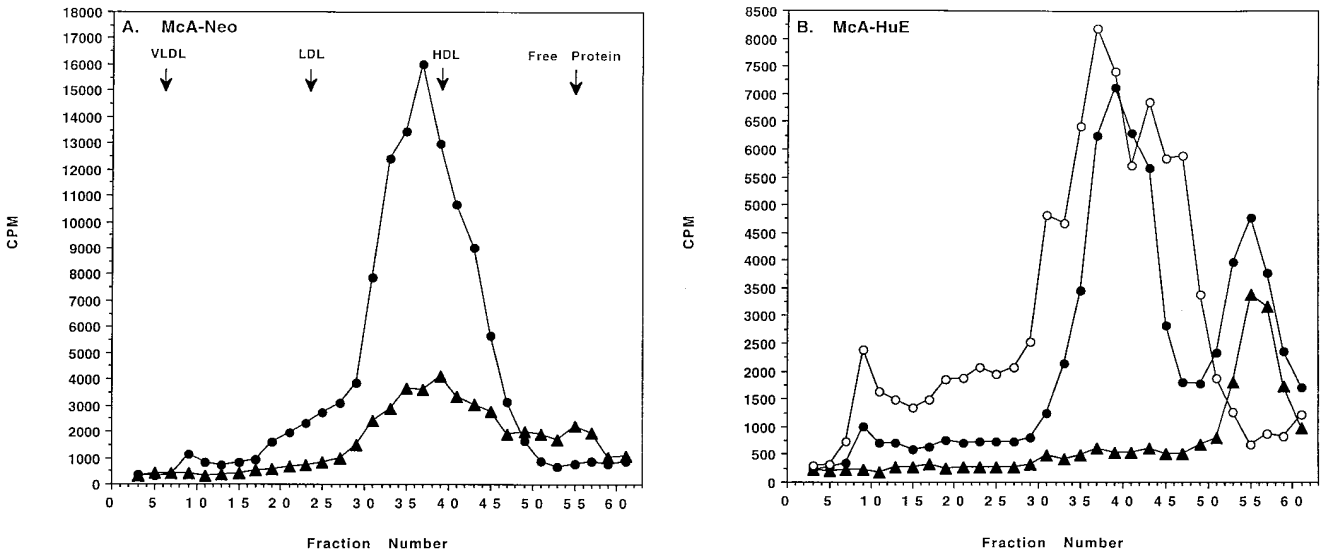

Fig. 2. Distribution of rat apolipoproteins and human apoE on media lipoproteins and in lipoprotein-free fractions separated via FPLC. McA-Neo-transfected (panel A) and McA-HuE-transfected (panel B) cells were grown in T75 flasks and treated with 1% LPDS as described in Experimental Procedures. After concentration, the media was fractionated on tandem Superose 6 columns and 50-μl aliquots of every other fraction were analyzed by Western blotting for rat apoA-I (▲), rat apoE (●), and human apoE (○) using species specific antibodies. After incubation with 1 μC of ^{125}I-labeled protein A and exposure to X-ray film, the radioactive bands corresponding to the specific apolipoproteins were excised, counted, and plotted. Representative graph; n = 10.

At 20×10^5 and 20×10^6 pfu per dish, apoA-I levels on the HDL particles were reduced by 44% and 68%, respectively, and there was an increase in the amount of apoA-I in the lipoprotein-free fractions (Fig. 3A). On the other hand, rat apoE levels in HDL were decreased only at the highest level of infection examined (Fig. 3B). At this level of infection a significant amount of human apoE was detected in the lipoprotein-free fractions (Fig. 3C), indicating that this level of expression of human apoE3 in the adenoviral infected cells was greater than that in the McA-HuE transfected cells.

Non-denaturing gels

The decrease in rat apoA-I associated with the HDL secreted by the cells expressing human apoE3 may be due to the displacement of apoA-I from apoA-I-containing nascent HDL particles by human apoE. Alternatively, apoE and apoA-I may be competing for a limited amount of lipid available for lipoprotein production. We reasoned that if the apolipoproteins were competing for the surface of the same lipoprotein particles, then it is possible that human apoE and rat apoA-I may be found on the same sized particles. On the other hand, if the two apolipoproteins are competing for available lipid, then the two apolipoproteins might occupy different size particles.

In order to obtain evidence on this postulate we examined the size of the lipoproteins containing the apolipoproteins by analyzing unfractionated media and HDL peak fractions from the FPLC and NaBr gradients on 4–30% non-denaturing gradient gels followed by Western blot analysis for the human apoE, rat apoE and rat apoA-I. Representative immunoblots are shown in **Fig. 4** and the mean radii of the particles containing each of the apolipoproteins is shown in **Table 2**. In the McA-Neo cells, particles with radii of 6.29 and 4.68 nm contained rat apoA-I. While rat apoE was also found on particles of radii 4.35 nm (not statistically different from 4.68 nm) rat apoE was found also on 5.28 and 6.71 nm particles. Thus, in McA-Neo cells the majority of the rat apoE and rat apoA-I are found on different sized particles. Human apoE associated with particles of similar size as rat apoE. The distribution of the rat apoE on the HDL particles was not dramatically altered upon expression of human apoE. While the results in Figs. 2 and 3 demonstrate that overexpression of human apoE3 displaces some rat apoE from the HDL particles, it was not possible to determine on the immunoblots of the non-denaturing gels whether there was a selective decrease in rat apoE associated with a particular size HDL particle. On the other hand, consistent with the results presented in Figs. 2 and 3, very little rat apoA-I was detected in the HDL particles secreted from the McA-HuE cells (data not shown).

Non-denaturing immunoprecipitations

The results of the immunoblots of the non-denaturing gels indicate that the HDL particles secreted from the transfected McA RH7777 cells are heterogeneous with respect to size and apolipoprotein distribution. But even in the instances when the apolipoproteins are detected on similar sized particles by the immunoblotting of non-denaturing gels, the apolipoproteins may occupy distinct particles. To further examine this we performed non-denaturing immunoprecipitations on [^{35}S]methionine-labeled HDL fractions separated on the FPLC columns (**Fig. 5A**). In Fig. 5B, the presence of apoA-I on apoE-containing particles was examined. When the HDL fractions from

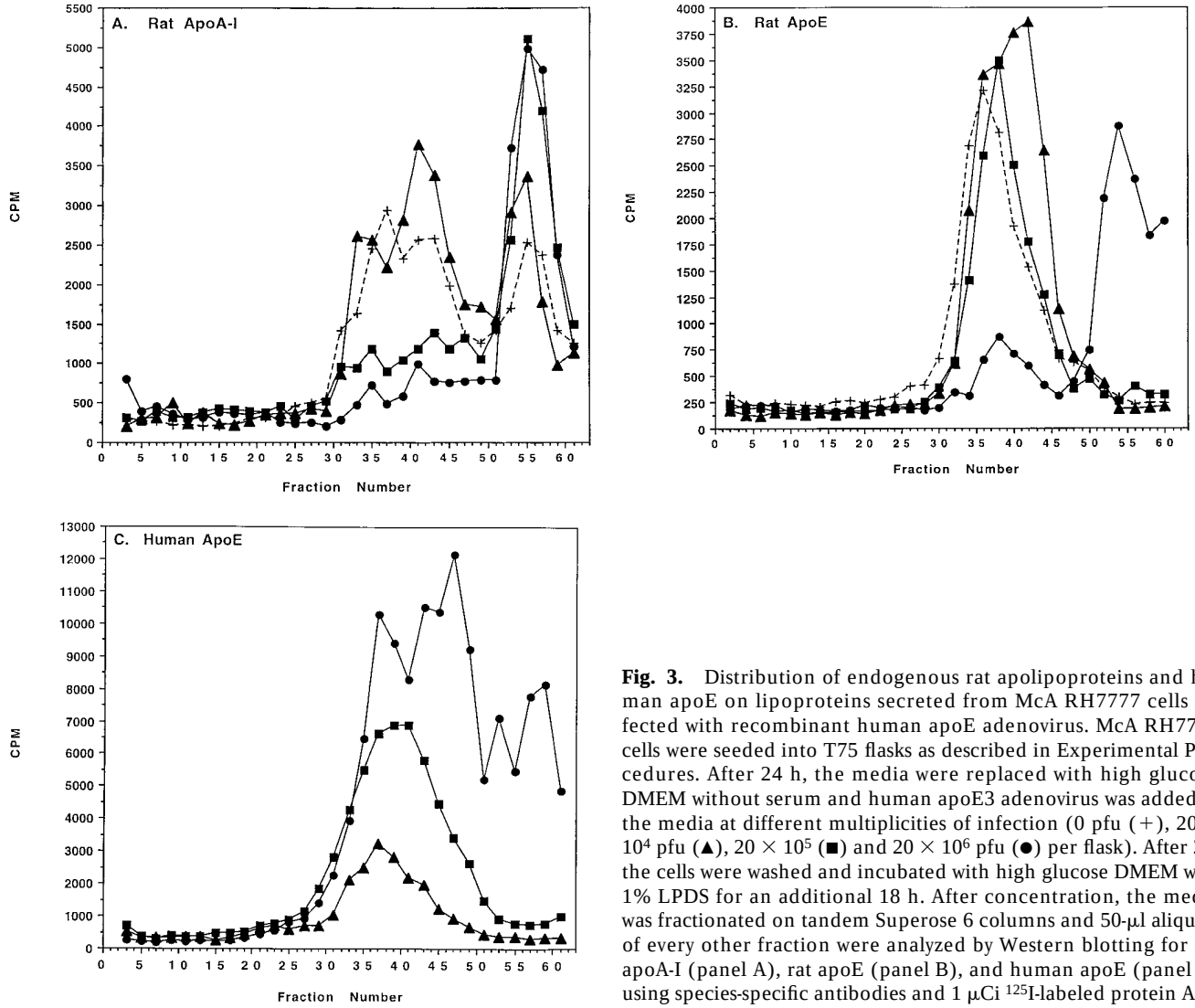

Fig. 3. Distribution of endogenous rat apolipoproteins and human apoE on lipoproteins secreted from McA RH7777 cells infected with recombinant human apoE adenovirus. McA RH7777 cells were seeded into T75 flasks as described in Experimental Procedures. After 24 h, the media were replaced with high glucose DMEM without serum and human apoE3 adenovirus was added to the media at different multiplicities of infection (0 pfu (+), 20×10^4 pfu (▲), 20×10^5 (■) and 20×10^6 pfu (●) per flask). After 2 h the cells were washed and incubated with high glucose DMEM with 1% LPDS for an additional 18 h. After concentration, the media was fractionated on tandem Superose 6 columns and 50-μl aliquots of every other fraction were analyzed by Western blotting for rat apoA-I (panel A), rat apoE (panel B), and human apoE (panel C) using species-specific antibodies and 1 μCi ^{125}I-labeled protein A.

McA-Neo cells were subjected to non-denaturing immunoprecipitations using rat apoE specific antibodies, very little rat apoA-I was co-precipitated (Fig. 5B, lane 1). Similarly, when the same fractions were immunoprecipitated with anti-rat apoA-I antibody, very little rat apoE was co-precipitated (Fig. 5B, lane 2). This confirms the suggestion from the non-denaturing gels that the majority of the rat apoE and apoA-I are located on different particles. With the HDL from the McA-HuE cells, no rat apoA-I was detected in the material immunoprecipitated by the hu-

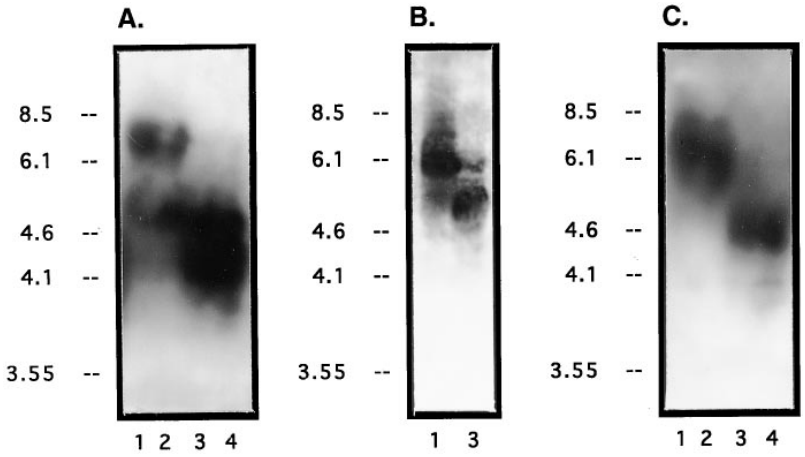

Fig. 4. Immunoblotting of non-denaturing gradient gels for human apoE. McA-HuE cells (panels A and B) or McA-Neo cells (panel C) were seeded in T75 flasks, treated with 1% LPDS, and the media were fractionated on tandem Superose 6 columns as described in Fig. 1. Aliquots (60 μl) of FPLC fractions 39 and 40 (lanes 1 and 2) and fractions 45 and 46 (lanes 3 and 4) were electrophoresed on 4–30% nondenaturing gradient gels, transferred to Immobilon-P membrane, and probed with human specific apoE antibodies (panel A), rat apoE specific antibodies (panel B), or rat apoA-I antibodies (panel C) followed by ECL detection. The following proteins were used to generate a standard curve to calculate the radii of the HDL particles: thyroglobulin, 8.5 nm; ferritin, 6.1 nm; catalase, 4.6 nm; lactate dehydrogenase, 4.1 nm; and albumin, 3.55 nm. Representative blots; n = 7.

TABLE 2. Radii of HDL particles containing rat apoA-I and rat or human apoE

Rat ApoA-I	Rat ApoE	Human ApoE
6.29 ± 0.16	6.71 ± 0.14[a]	6.47 ± 0.10
	5.28 ± 0.10	5.14 ± 0.16
4.68 ± 0.13	4.35 ± 0.12	4.33 ± 0.07

Total media or HDL fractions from the FPLC or NaBr equilibrium gradients were applied to 4–30% non-denaturing gradient gels and immunoblotted for the various apolipoproteins using species specific antibodies as described in Fig. 4. The differences in the radii were analyzed by ANOVA analysis; n = 7

[a] Significantly different from rat apoA-I 6.29 nm particles, $P < 0.05$. Radii of particles on lines 2 and 3 are not significantly different from each other.

man apoE specific antibodies (Fig. 5B, lane 3), however a small amount of rat apoA-I was immunoprecipitated with the rat apoA-I antibody (Fig. 5B, lane 4). The human apoE radioactivity observed in lane 4 is due to non-specific absorption as the amount is equivalent to the amount of non-specific absorption of human apoE obtained when these fractions were immunoprecipitated with anti-human apoA-I specific antibodies that do not cross-react with rat apoA-I (data not shown). Thus, both rat and human apoE proteins secreted from the McA RH7777 cells are found on particles that contain very little, if any, apoA-I.

Immunoblotting of non-denaturing gels (Table 2) suggested that rat and human apoE are found on similar size HDL particles secreted from the transfected cells. This was examined further in Fig. 5C. As human apoE and rat apoE cannot be resolved by electrophoretic mobility on SDS-polyacrylamide gels, it was necessary to subject the proteins precipitated by the non-denaturing immunoprecipitation to a subsequent denaturing immunoprecipitation with human and rat specific antibodies in order to determine whether both of these apolipoproteins can be found on the same particles. The results of these double immunoprecipitations indicate that the HDL particles secreted from the McA-HuE cells appear to contain primarily rat or human apoE with very few particles (<25%) containing both apolipoproteins.

In an attempt to determine whether differences in the lipid composition of the HDL particles accounts for the fact that rat apoE, rat apoA-I, and human apoE are not found on the same HDL particles, the cells were labeled with [^{3}H]glycerol or [^{3}H]cholesterol. The HDL particles were isolated by FPLC, the pooled HDL peaks were subjected to non-denaturing immunoprecipitation using non-immune serum or antibodies to the rat or human proteins, and the labeled lipids in the precipitated HDL were analyzed by thin-layer chromatography (**Table 3**). The total HDL pool from McA-HuE cells contained 2 to 3 times more phospholipid (PL) and free cholesterol (FC) than the pool from the McA-Neo cells, most likely reflecting the overexpression of human apoE in these cells. In both the total HDL pool and in the immunoprecipitated HDLs, the majority (>76%) of the labeled glycerol was incorporated into PL, while the majority of the labeled cholesterol (>90%) was FC. The relative deficiency in triglycerides (TG) and cholesteryl esters (CE) is consistent with a small hydrophobic core in the HDL particles. There was no detectable difference in the labeled lipid composition of the HDLs immunoprecipitated with each of the specific antibodies. The major PL species in the HDL particles was phosphatidylcholine (data not shown).

DISCUSSION

In this paper, we have characterized the lipoproteins secreted by McA RH7777 cells transfected with a neomycin resistant marker (control) and those overexpressing human apoE3. The majority of the rat apoE, rat apoA-I, and human apoE secreted from the transfected cells were associated with the HDL particles, although a small amount of both rat and human apoE, but not rat apoA-I, were detected in the VLDL fractions. This is consistent with the results of Fazio and Yao (31) who have shown that in the absence of serum the majority of apoE is asso-

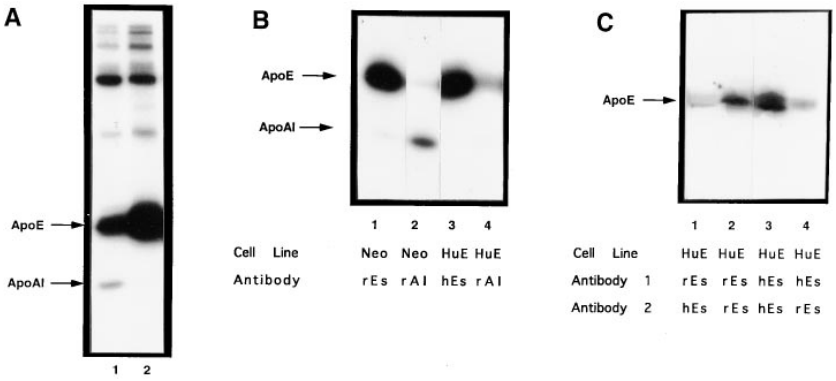

Fig. 5. Non-denaturing immunoprecipitation of HDL fractions. Transfected McA RH7777 cells (McA-Neo and McA-HuE) were seeded into T-75 flasks, labeled with Tran^{35}S-label in the presence of 1% LPDS, and the media were fractionated on FPLC columns as described in Experimental Procedures. HDL fractions were pooled, adjusted to 0.2 m NaCl, and subjected to non-denaturing immunoprecipitation using anti-rat apoE specific antibodies (rEs), anti-human apoE specific antibodies (hEs), or anti-rat apoA-I antibodies (rAI). Panel A: The pooled HDL fractions from McA-Neo (lane 1) and McA-HuE (lane 2) cells. The arrows indicate the mobility of rat/human apoE and rat apoA-I. Panel B: The HDL fractions were subjected to non-denaturing immunoprecipitation with the indicated antibodies. An 18-h exposure was used in order to detect rat apoA-I. Panel C: The McA-HuE HDL fractions were first subjected to non-denaturing immunoprecipitation with antibody 1 and the immunoprecipitated proteins were solubilized in SDS/Triton X-100/deoxycholate, and subjected to denaturing immunoprecipitation with the indicated antibody 2 as described in Experimental Procedures.

TABLE 3. Incorporation of [^{3}H]glycerol and [^{3}H]cholesterol into HDL particles

A. Total HDL

Cell Line	PL	TG	FC	CE
	cpm/mg cell protein			
McA-Neo	684	216	5002	362
McA-HuE	1492	214	17524	361

B. PL and FC in HDL isolated by non-denaturing immunoprecipitation

	McA-Neo			McA-HuE		
	rE	rAI	hE	rE	rAI	hE
	cpm/mg cell protein					
PL	363	194	—	118	180	316
FC	356	403	—	711	457	2658

Cells were labeled with [^{3}H]glycerol or [^{3}H]cholesterol as described in Experimental Procedures. The HDL was isolated by FPLC and the peak HDL fractions were pooled and subjected to non-denaturing immunoprecipitatioin with non-immune serum, anti-rat apoA-I (rAI) or antibodies specific for rat (rE) or human (hE) apoE. The lipids in aliquots of the pooled HDL (A) and in the immunoprecipitates (B) were extracted and separated by TLC, and counts in the phospholipids (PL), triglycerides (TG), free cholesterol (FC), and cholesteryl ester (CE) spots were expressed as cpm/mg cell protein.

ciated with HDL particles secreted from McA RH7777 cells and that it becomes redistributed to apoB-containing particles in the presence of serum. In our experimental protocol the cells was incubated in the absence of serum lipoproteins and this most likely accounts for the low level of association of apoE with VLDL particles. Thus, we focused on the differential association of apoE and apoA-I with the HDL particles secreted from the transfected McA RH7777 cells. The most significant observations of this study are as follows. *a*) apolipoproteins A-I and E are found on two or three different size HDL particles; *b*) the majority of the rat apoA-I and apoE is found on different particles in the HDL class; *c*) human apoE expressed by transfected rat hepatoma cells reduces the association of rat apoA-I with secreted HDL, with the result that more of the latter apolipoprotein is found in the lipoprotein-free fraction; *d*) high level overexpression of human apoE results in the secretion of a significant proportion of this apolipoprotein in a lipoprotein-free or lipid-poor fraction; and *e*) the lipoprotein particles contain a limited hydrophobic core. From these results, we conclude that the size and composition of nascent lipoprotein is heavily influenced by the nature and amount of the apolipoproteins produced by the secreting cells, and probably by the lipid available for packaging of secreted lipoproteins.

Rat apoA-I and apoE occupy different HDL particles

Our results indicate that the HDL particles secreted from the McA RH7777 cells are heterogeneous with respect to size and apolipoprotein composition. Multiple discrete particles with distinct radii could be detected on non-denaturing gels. The non-denaturing gels (Table 2) and the non-denaturing immunoprecipitations (Fig. 5) indicate that the majority of these two rat apolipoproteins occupy different HDL particles. Particles from the McA-Neo cells with radii <4.7 nm appeared to contain both rat apoE and apoA-I. The low levels of particles containing both rat apoE and rat apoA-I obtained by non-denaturing immunoprecipitation using antibodies to either of these apolipoproteins suggest that the particles with radii <4.7 nm represent a minor fraction of the total HDL particles and that the majority of these two apolipoproteins is located on unique particles with radii >4.7 nm. Previous studies have shown that McA RH7777 HDL lipoproteins containing rat apoA-I have exclusively α-mobility on agarose gels (22, 32), while apoE was detected on α-migrating particles and particles with pre-β-like mobility (31). Transfected rat apoA-IV was also associated with discrete HDL particles that were deficient in apoA-I (22). Negative staining electron microscopy of HDL particles secreted from Neo transfected cells into serum-free media performed by Yao et al. (22) demonstrated that the HDL particles were primarily spherical. Assuming that these apolipoproteins are primarily α-helical and that the area occupied by each amino acid in an α-helix is 1.5 nm, two or more molecules of rat apoE and apoA-I could be accommodated on spherical particles with radii >4.7 nm. Despite this, the rat apolipoproteins seldom appear to occupy the same particle, whether in the same size range or not. The largest rat apoE-containing particle is significantly larger than the largest apoA-I-containing particle (6.71 nm vs. 6.29 nm). These observations are consistent with a number of prior reports involving the study of the lipoproteins secreted by perfused primate liver (33, 34), by perfused rodent liver (35), by hepatocytes (36), or the study of recombinant particles (37) where it was shown that apoE is associated with larger particles than apoA-I.

Properties of HDL-like lipoproteins secreted by hepatoma cells overexpressing wild type human apoE

In many respects, human apoE behaved like rat apoE. Like endogenous rat apoE, human apoE was distributed among three different sizes of HDL. In no case was there a significant difference in the size of the particles with which rat and human apoE associated. However, the non-denaturing immunoprecipitation experiments revealed that rat and human apoE seldom coexisted on the same particles. This unexpected result may relate to the tendency of the amphipathic apolipoproteins to associate with one another and suggest that homologous apolipoproteins may associate more readily with proteins of the same sequence than with proteins whose sequences differ even modestly (in this case, rat and human apoE). It is not clear how much of the distribution pattern we observed is attributable to the 2.6-fold higher level of expression of the human homologue.

The most striking property of the overexpression of human apoE is the displacement of rat apoA-I from the HDL fractions. This occurs without any significant impact on the total amount of rat apoA-I secreted by the hepatoma cells (Table 1). It is clear from the non-denaturing immunoprecipitation of intact particles that apoE, whether rat

or human, occupies different particles than apoA-I. The most likely explanation for our findings is that there is a limiting amount of phospholipid that forms the surface of secreted lipoproteins, and that overexpression of either apolipoprotein A-I or E would result in a proportion of the apolipoprotein being secreted in a lipid-poor state. Prior in vivo results are compatible with this possibility. Davis et al. (38) showed that in starved rats, a larger than normal proportion of apoE was in a lipid-poor state. This may also account, in part, for the effect of overexpression of human apoA-I in transgenic mice on the fate of murine apoA-I (39). Transgenic models involving overexpression of human apoE have not been carefully evaluated for the composition of the HDL fractions (40, 41). It is not possible to determine the relative avidity of apolipoproteins for the lipoprotein surface from our experiments. We did not evaluate the absolute amounts of apolipoproteins E and A-I produced by these cells. However, from the fluorograph of total HDL proteins (Fig. 5A), it appears that apoE is produced at a higher level than apoA-I. When human apoE is overexpressed, a much larger proportion of apoA-I is displaced to the lipid-poor fraction than is rat apoE. This might be taken to indicate that apoE has a higher avidity for these lipoprotein surfaces while apoA-I has the lowest avidity among rat apoE, human apoE, and rat apoA-I. Careful in vitro competition experiments for a standard lipoprotein surface would be needed to confirm this suggestion.

In addition, we have noted that the recovery of rat apoA-I from FPLC and NaBr equilibrium single spin gradient fractionation of McA-HuE cell media is not complete. We have carefully examined the media from McA-Neo and McA-HuE cells for rat apoA-I at each step in the preparation of the samples. Both samples have comparable amounts of apoA-I up to the point when they are applied to the FPLC columns or the equilibrium density gradients. This suggests that the rat apoA-I in the McA-HuE media is being lost or adhering to the columns or tubes during fractionation. This further supports our conclusion that the association of rat apoA-I with lipoprotein particles is altered when human apoE is expressed by the rat hepatoma cells.

The nature of the nascent lipoprotein

Prior studies of the lipoproteins secreted by McA RH7777 cells has suggested that small, spherical particles are secreted (22). This is based upon negative staining electron microscopy. Our results are not fully in conformity with these prior conclusions. The non-denaturing gradient gel electrophoresis argues that the bulk of the apolipoproteins E and A-I are found on particles of radii of about 6.5 nm, which is not small for HDL particles. Yet the labeling of the secreted lipids with either cholesterol or glycerol indicates that the core lipoprotein lipids, triglycerides and cholesteryl esters, are present in very modest proportion. We have previously observed a non-discordance between non-denaturing gel electrophoresis and negative staining electron microscopy for serum amyloid A-containing HDL (42). We have suggested that this apparent discrepancy may be explained by an irregular hydrophilic surface that is hydrodynamically active in the non-denaturing gels, but can be penetrated to the small hydrophobic core by the negative stain used for electron microscopy.

In conclusion, this study indicates that the size and composition of nascent secreted lipoproteins are highly determined by the properties of the secreted apolipoproteins, and that a dominant apolipoprotein is a determinant. These conclusions may not be fully applicable to the association properties of apolipoproteins with mature lipoproteins refashioned by lipid metabolism in the plasma. Further experiments are in progress to explore this latter relationship.

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