The HepG2 Extracellular Matrix Contains Separate Heparinase- and Lipid-releasable Pools of ApoE

IMPLICATIONS FOR HEPATIC LIPOPROTEIN METABOLISM

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We have examined the association of apoE with the extracellular matrix (ECM) of HepG2 cells. Comparison of ECM prepared by previously published methods demonstrated that cytochalasin B-prepared material yielded the highest endogenous apoE, representing 23.6% of that in cell monolayers. ECM prepared with EDTA or Triton X-100 exhibited decreased levels of apoE, 3 and 6%, respectively. ECM bound very low density lipoprotein poorly (5–6% of the monolayer capacity); however, these incubations dramatically increased the apoE content of the ECM. Heparinase or suramin decreased apoE of the ECM by 19.6 and 37.3%, respectively, suggesting association with heparin sulfate proteoglycans. EDTA or EGTA also displaced 35% of the apoE, suggesting a Ca2+-dependent association. Incubation with phosphatidylcholine vesicles (PCV) displaced 30% of the apoE, suggesting that lipid content affects association of apoE with the ECM. Data derived from sequential incubations with combinations of suramin, EGTA, and PCV were consistent with the presence of two distinct pools of apoE on the HepG2 ECM, one releasable with suramin and EGTA and the other releasable with lipids. Exogenously applied lipid-free apoE readily bound to the ECM; however, increasing the lipid content decreased its association. Lipid-free apoE could be equally displaced from the ECM with PCV or suramin. When lipid-free apoE adsorbed to microtiter wells was incubated with a triglyceride emulsion or palmi

In the liver, the abundant gaps and fenestrations of the sinusoidal endothelial cells form an open network that allows an efficient exchange of macromolecules between the blood and hepatocytes (1). The endothelium is separated from the hepatocyte plasmalemma by the space of Disse that contains irregular clumps of extracellular matrix (2). The ECM is the basal lamina secreted by cells and on which cells migrate, proliferate, and differentiate in vivo (3). Several studies have contributed to the characterization of the ECM, which is defined in vitro as the growth substratum remaining attached to culture dishes after removal of cells by Triton X-100 (3–5), EDTA (5), or cytochalasin B (6). Major components of the hepatic ECM include collagen type III, collagen IV, fibronectin, and laminin (2, 7, 8) as well as abundant basement membrane-type heparin sulfate proteoglycans (HSPG) (7, 9, 10). In the liver, the ECM is a discontinuous structure and is therefore unlikely to have an efficient filtration function as described for more organized matrices of other tissues (11). A potential function for the hepatic ECM, suggested by the presence of HSPG, could involve a role in lipoprotein metabolism. Lipoprotein lipase readily interacts with HSPG of the ECM elaborated by bovine corneal endothelial cells (12) or aortic endothelial cells (4, 13). Other components of chylomicron remnant and VLDL remnant metabolism, including hepatic lipase (14, 15), apolipoprotein E (apoE) (16–20), as well as apoE-enriched remnant particles (18, 20, 21), bind to HSPG.

ApoE secreted by hepatocytes into the space of Disse is believed to participate in the secretion-capture pathway of lipoprotein metabolism (18, 20). ApoE enrichment of lipoproteins increases the affinity of the lipoprotein for HSPG and the LRP (18, 20) and hence increases the surface binding and internalization of lipoproteins. ApoE is also associated with the basolateral surface of hepatic parenchymal cells (22), as well as the surface of HepG2 cells (16, 17) and McA7777 cells transfected with human apoE3 (18). In these latter studies, incubation of cells with heparin, suramin, or heparinase increased the apoE content of the surrounding medium, suggesting attachment with the cell surface via HSPG. Cell surface apoE appears to be internalized upon perfusion of the liver with chylomicron...
remnants (21), suggesting a role in lipoprotein internalization.

In the present study, we have measured the specific binding of radiolabeled antibodies against apoE to the ECM of HepG2 cells to directly resolve the nature of the relationship between apoE and the ECM. Our results suggest that significant amounts of endogenous apoE associate with the ECM through HSPG-, divalent metal ion-, and lipid-dependent interactions. We provide evidence that the HSPG- and EGTA-releasable pools of apoE represent a single population that is distinct from a lipid-releasable pool. We also demonstrate that apoE of the ECM is lipid-poor and dissociates with the addition of lipid. This property could allow ECM apoE to participate either in lipoprotein secretion or in lipoprotein uptake.

**EXPERIMENTAL PROCEDURES**

**Materials**

Na\(^{125}\)I was obtained from Amersham Corp. (Oakville, Ontario, Canada). IODO-BEADs were from Pierce. EDTA was obtained from British Drug House. Cytochalasin B, heparin from pork intestinal mucosa, suramin, heparinase type I, EGTA, egg phosphatidylcholine, and most other chemicals were obtained from Sigma. POPC and DMPC were obtained from Avanti Polar Lipids (Birmingham, AL). Liposyn II, a solubilized triglyceride-rich emulsion containing 10% safflower oil, 10% soybean oil, 1.2% egg phospholipids, and 2.5% glycerin is a product of Cosa, suramin, heparinase type 1, EGTA, egg phosphatidylcholine, and Canada). IODO-BEADs were from Pierce. EDTA was obtained from Iodine Foundation Laboratories for Cardiovascular Disease, University of California, San Francisco). Rabbit polyclonal anti-human LRP was a generous gift from Dr. Guojun Bu (Washington University School of Medicine). Iodination of antibodies was performed by the IODO-BEAD method (Pierce), and specific activities from 1.5 to 10.6 \(\mu\)Ci/\(\mu\)g were obtained.

**Lipoproteins and Lipids**

Fresh plasma was obtained from the Canadian Red Cross or from healthy normolipemic volunteers, from which lipoproteins were isolated by density gradient centrifugation as described by Rall et al. (27). After extensive dialysis against PBS, protein determinations were done by the Markwell Lowry method (28). VLDL was iodinated by the IODO-BEAD method (Pierce). ApoVLDL was prepared as previously reported (27).

Spherical particles were produced from egg phosphatidylcholine by the sonication method of Sparks et al. (30). POPC cholate micelles were prepared as described by Sparks et al. (30).

**Cell Culture**

HepG2 cells were cultured in EMEM growth medium containing 10 mM HEPES, 10% fetal bovine serum, 2 mM glutamine, penicillin (100 units/ml), and streptomycin (100 \(\mu\)g/ml). Confluent cultures were split 1:6 to 1:8 (100,000 cells/cm\(^2\)) into 12-well plates for individual experiments. On the day prior to surface labeling experiments, fresh medium was added to the cells. All experiments were done on fully confluent cultures.

**Preparation of Extracellular Matrix**

Extracellular matrix was prepared from fully confluent HepG2 cell monolayers by three methods. All ECM preparations were washed three times with ice-cold PBS-BSA prior to experiments.

**Method 1—ECM** was prepared by the Triton X-100 method of Owensby et al. (5). Briefly, the medium was aspirated from HepG2 cells and replaced with 1% Triton X-100 in PBS containing 1 mM phenylmethylsulfonyl fluoride. The cells were incubated at 37°C for 5 min and then the solubilized cells were aspirated off and replaced with ice-cold PBS containing 2 mg/ml of fatty acid-free BSA and 1.7 mM Ca\(^{2+}\) (BSA).

**Method 2**—ECM was prepared by incubation of HepG2 cell monolayers with PBS containing 3 mM EDTA and 1 mM phenylmethylsulfonyl fluoride for 5 min at 37°C as described by Owensby et al. (5).

**Method 3**—ECM was prepared from HepG2 monolayers by a modification of the cytochalasin B method of Mai and Chung (6). Briefly, 10 \(\mu\)l of a 1 mg/ml stock of cytochalasin B in ethanol was added directly to the HepG2 cell monolayer, and the complete medium to a final concentration of 10 \(\mu\)g/ml. The cells were incubated for 60 min at 37°C and then transferred to ice. The cell monolayers lifted off the plates as sheets of cells when subjected to repeated aspirations of medium with a P1000 pipette.

**Determination of the ApoE Content of the Cell Surface and ECM**

The apoE content of cell monolayers or ECM was determined using radiolabeled 7C9. This monoclonal antibody recognizes an N-terminal epitope of apoE and provides determinations of ECM apoE that are independent of lipid content (see below). Cells were prechilled for 15 min on ice and then washed three times in ice-cold PBS-BSA. Iodinated mAb (5 \(\times\) 10\(^5\) cpm/ml), diluted in PBS-BSA, was added to wells containing either cell monolayers or ECM and incubated at 4°C for 2–4 h. To determine nonspecific binding, a 100-fold excess of cold mAb was added to replicate wells. After incubation, the cells or ECM were washed three times in cold PBS containing 1.7 mM Ca\(^{2+}\) and then solubilized with 1 ml of 0.5 M NaOH. Radioactivity was measured in a \(\gamma\) counter, and the results for antibody binding are expressed as the mean specific binding/well. Experiments were always performed in triplicate for both total and nonspecific binding in multi-well plates.

In some studies, radiolabeled 3H1 was used to characterize apoE of the cell surface or ECM. In these experiments, ECM or HepG2 cell monolayers were first incubated at 4 or 37°C for 1 h in the absence and presence of 1 mg of Liposyn II or POPC micelles/ml. The lipids were removed by two washes with PBS-BSA, and the incubations with radiolabeled 3H1 were performed at 4°C as described above for 7C9. Competition Scatchard analysis was performed on cell monolayers that had been preincubated in the absence or presence of 1 mg/ml Liposyn II. These fractions were incubated with a constant amount of \(^{125}\)I-mAb and a range of concentrations of the same unlabeled mAb in triplicate wells for each point.

**Lipoprotein Binding to Cell Monolayers and ECM**

HepG2 cells or ECM were incubated at 4°C for 30 min in PBS-BSA. Human VLDL (100 \(\mu\)g/ml, final concentration) was then added, and the incubations were continued at 4°C until an additional 1 h at 4°C had elapsed. Lipoproteins were removed with three washes with PBS-BSA. VLDL particles bound to the cell surface or ECM were detected by the addition of radiolabeled 1D1 or 7C9 (500,000 cpm/ml). Nonspecific binding was determined in the presence of a 200-fold excess of unlabeled antibody. Antibody incubations were continued for 2–4 h at 4°C. Unbound antibody was removed with three washes with ice-cold PBS, and then the cell fractions were solubilized with 0.5 N NaOH. Radioactivity was measured in a \(\gamma\) counter, and the results were expressed as the mean specific antibody binding/well.

Specific VLDL binding to cell monolayers or ECM was also determined by the addition of \(^{125}\)I-VLDL (500,000 cpm/ml) to PBS-BSA in the absence and presence of a 200-fold excess of unlabeled VLDL. These incubations were conducted at 4°C for 2 h and were followed by three washes with ice-cold PBS-BSA and solubilization with 0.5 N NaOH. Radioactivity was measured in a \(\gamma\) counter, and the results were expressed as the mean specific VLDL binding/well.

**ApoE Lipid Content and Immunoreactivity of Anti-apoE Monoclonal Antibodies**

A solid phase radioimmunoassay was used to examine the effect of apoE lipidation on the immunoreactivity of monoclonal antibodies. Briefly, 0.5 \(\mu\)g of lipid-free apoE or 5 \(\mu\)g of VLDL in 100 \(\mu\)l of PBS was immobilized by passive adsorption to 96-well microtiter plates for 16 h at 4°C. Unbound material was removed with three washes of PBS, and then the wells were incubated with 100–\(\mu\)l volumes of Liposyn II or POPC cholate micelles (0–0.4 \(\mu\)M) in PBS for 2 h at 37°C. The wells were then washed three times with PBS and blocked with 300 \(\mu\)l of PBS containing 1% fatty acid-free BSA for 2 h at 20°C. \(^{125}\)I-labeled anti-
apoE antibody (100,000 cpm/well) was added and incubated for 2 h at 20 °C. The wells were then washed three times with PBS-BSA and counted.

**Electrophoresis and Western Blotting**

ECM was prepared from confluent monolayers grown in T-175 cm² flasks (2 flasks/matrix preparation) as described above. 7 ml of Laemmli sample buffer (31) containing SDS (2%) and 2-mercaptoethanol (5% w/v) was added to each T-175 flask and allowed to solubilize the matrix overnight at 25 °C with gentle rocking. The samples were concentrated approximately 5-fold (final volume 2.8 ml) in Centriprep 10 units (Amicon) and then heated at 95 °C for 5 min. Cell or ECM proteins were separated by SDS-polyacrylamide gel electrophoresis (7.5% separating gel, 4% stacking gel) and then transferred to nitrocellulose membranes (0.45 μm, Bio-Rad). PBS containing powdered milk (3% w/v) was used to block the nitrocellulose membranes and to dilute antibody stocks.

The nitrocellulose membranes were incubated with gentle rocking for 1 h at 25 °C with rabbit antiserum specific for human fibronectin (Sigma), human apoE, or human LRP followed by incubation for 1 h with goat anti-rabbit IgG coupled to horseradish peroxidase. Peroxidase activity was detected using the chemiluminescence method of Boehringer Mannheim.

**RESULTS**

**Determination of ApoE of the ECM and HepG2 Cell Surface**—Previous reports have characterized cell surface apoE indirectly by measuring increases in medium apoE following incubation of cells with heparin or heparinase at 37 °C (16–18). These results are likely to be complicated by secretion of apoE. In this study, we have assessed cell surface apoE directly by determining the specific binding of radiolabeled monoclonal antibodies against apoE to the cell surface or to the ECM of HepG2 cells. Monoclonal 7C9, which recognizes residues 1–15 of the N-terminal domain of apoE, was incubated with HepG2 monolayers at 4 °C to minimize membrane turnover and secretion. Fig. 1 demonstrates that there is significant and specific binding of ¹²⁵I-7C9 to the HepG2 cell surface (top panel). In contrast a monoclonal IgG against an irrelevant antigen (atrial natriuretic factor) displayed no specific binding to HepG2 cells (data not shown). Suramin promoted a 36% decrease in the apparent binding of ¹²⁵I-7C9 to the cell surface (top panel). The decreased 7C9 binding observed after suramin incubations results from displacement of apoE from cell surface HSPG and is paralleled by a 2-fold increase in the amount of apoE in the surrounding media (bottom panel). Control experiments have shown that suramin exerts no effect on the apoE radioimmunoassay (not illustrated). Collectively the data argue that the specific binding of 7C9 to the cell surface accurately reflects the levels of cell surface apoE.

**Characterization of the HepG2 Cell ECM**—We reasoned that the ECM and its complement of HSPG should interact with secreted apoE and present a simplified system to study the interaction of apoE with extracellular components. Consequently, ECM was prepared by three previously published methods that utilize incubations with 3 mM EDTA, 1% Triton X-100, or 100,000 cpm/well) was added and incubated for 2 h at 20 °C. The wells were then washed three times with PBS-BSA and counted.

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The apolipoprotein E content of ECM prepared by different methods

| ECM preparation | $^{125}$I-7C9 bound | Recovery of apoE |
|-----------------|----------------------|------------------|
| ECM Triton X-100 | 720 ± 144            | 6.0              |
| EDTA            | 357 ± 6.4            | 3.0              |
| Cytochalasin B  | 2822 ± 869           | 23.6             |
| Cell-free       | 28 ± 16              |                  |

ApoE and Lipoprotein Binding by the ECM

Confluent cell monolayers or ECM prepared by the Triton X-100 or cytochalasin B methods were washed twice with PBS-BSA and then incubated with $^{125}$I-7C9 (500,000 cpm/well, representing 70–100 ng of 7C9 protein) for 2 h at 4 °C. The cells were then washed three times with PBS containing 1.7 mM CaCl$_2$, solubilized overnight in 0.5 N NaOH, and counted in a γ counter. All data are the means and S.E. of three separate experiments.

Relative increases in apoE and apoB content of the HepG2 cell surface and ECM after incubation with conditioned medium or human VLDL

| Relative change after conditioned medium | VLDL |
|-----------------------------------------|------|
| + 100 µg VLDL                           | 0.36 |
| Absolute change after VLDL              | 10908|

| Anti apoE 7C9 | Anti apoB 1DI |
|---------------|---------------|
| cpm/well      | cpm/well      |
| Cell monolayer|                |
| + conditioned medium | 11713 ± 694 | 717 ± 57 |
| Absolute change after VLDL | 10908 | 8365 |
| ECM, Triton X-100 method | 872 ± 236 | 85 ± 44 |
| + conditioned medium | 3498 ± 623 | ND |
| Absolute change after VLDL | 12941 | 403 |
| ECM, cytochalasin B method | 3450 ± 348 | 187 ± 36 |
| + conditioned medium | 7627 ± 1794 | ND |
| Absolute change after VLDL | 5251 | 383 |

| Cell-free wells |                |
| + conditioned medium | 44 ± 12 | 145 ± 16 |
| + 100 µg VLDL | 343 ± 224 | 123 ± 45 |

* ND = not done.

protein.

Apolipoprotein E Content of the ECM and HepG2 Cell Surface—Table I compares the binding of 7C9 to the HepG2 cell surface and the ECM prepared by the three methods. The ECM preparation protocols yield matrices with significantly different abilities to bind 7C9, in descending order are cytochalasin B ECM > Triton X-100 ECM > EDTA ECM. In contrast, cell free wells treated in an identical manner retained only background levels of radiolabeled antibody. Matrix prepared by the cytochalasin B method bound $^{125}$I-7C9 to levels representing 23.6% of that bound by HepG2 cell monolayers. The Triton X-100 and EDTA preparations represented only 6.0% and 3.0% of the monolayer capacity, respectively. The ECM preparations and whole cell homogenates were also analyzed by Western blotting with anti-human apoE (Fig. 2C). In the whole cell homogenates, two bands in the 32–34 kDa range are readily visible and represent sialylated (Fig. 2C, upper band) and non-sialylated forms of apoE (32, 33). Both bands are visible in the ECM preparations although the upper sialylated band of apoE predominates. Band intensity is greatest in the cytochalasin B-prepared material, indicating, in agreement with the $^{125}$I 7C9 binding analysis of Fig. 1, that this method of preparation yields matrix with the highest endogenous apoE content. The variable 7C9 binding activity of the preparations could be explained by a differential loss of apoE during the procedures or by reduced binding of 7C9 due to conformational changes in apoE brought about by ECM preparation. We first tested the effects of the matrix preparation procedures on apoE that had been exogenously bound to the ECM. To minimize the concentration of endogenous apoE, we used apoE-poor Triton X-100 ECM. Incubation of Triton X-100-derived ECM with conditioned medium at 4 °C results in a 4-fold increase in the 7C9 binding activity (Table II). The apoE-enriched ECM were then subjected to one of the three preparation protocols and a PBS control, washed twice with PBS-BSA, and then incubated with $^{125}$I-7C9 (Fig. 3). The EDTA and Triton X-100 protocols reduced the $^{125}$I-7C9 binding activity by 70 and 40%, respectively. In contrast to these harsh extraction procedures, the cytochalasin B protocol did not decrease the apoE content of the ECM relative to control incubations. The recoveries of exogenous apoE during the preparation protocols closely paralleled those of endogenous apoE. In Table I. We also tested the effects of the preparation protocols on the ability of 7C9 to recognize apoE. We subjected apoVLDL, immobilized in microtiter wells, to the matrix preparation conditions and a PBS control and found no change in the subsequent binding of $^{125}$I-7C9 to apoE following the EDTA and cytochalasin B protocols. This type of analysis was not possible for the Triton X-100 preparation method since we found that incubation with this protocol resulted in a 77.3% loss of immobilized and radiolabeled apoE from the microtiter wells. There was, however, a coincident 75% decrease in the binding of $^{125}$I-7C9 to the microtiter wells. Thus the decreased binding of 7C9 with prior Triton X-100 treatment correlates best with the amounts of immobilized apoE rather than alterations in the immunoreactivity of 7C9 for apoE as a result of Triton X-100-induced conformational changes. These results indicate that the 7C9 binding activity of the matrix preparations reflects the variable recovery of endogenous apoE rather than conformational alterations of apoE brought about by the preparation procedures. With regard to the apoE content, the cytochalasin B-derived ECM appears to be the best representative of the native ECM of HepG2 cells because this preparation method yields the highest endogenous apoE content and does not result in the loss of exogenously added and bound apoE-containing lipoproteins.

Lipoprotein Binding by ECM Prepared by Different Methods—To determine the capacity of ECM for binding apoE-containing lipoproteins relative to HepG2 cell monolayers, we incubated the Triton X-100- and cytochalasin B-derived preparations of ECM and the HepG2 cells with conditioned medium or 100 µg of human VLDL at 4 °C. The unbound lipoproteins were removed with two washes in PBS-BSA, and then the ECM and cells were probed with radiolabeled 7C9 (Table II). This protocol thus assesses the total contributions of endogenous and exogenously applied apoE to the cell fractions. Following incubation with conditioned medium, the binding of $^{125}$I-7C9 to ECM prepared by the Triton X-100 and cytochalasin B methods increased 4- and 2.2-fold, respectively. A second incubation
with conditioned medium did not further increase the apoE content of the ECM, indicating that saturation had been achieved. The conditioned medium used in these studies was derived from a 72-h incubation of a confluent HepG2 monolayer and is relatively more conditioned than the media (24 h incubation) bathing the monolayers just prior to our experiments. Therefore, with our standard cell culture conditions for the ECM preparation protocols, the cell surface and ECM are not fully saturated with apoE. Most striking are comparisons between the levels of 7C9 binding achieved after incubation of ECM and monolayers with VLDL. Under these conditions, 7C9 binding to the HepG2 monolayers increased by 1.9-fold. In contrast, 7C9 binding to the Triton X-100- and cytochalasin B ECM, the binding of intact VLDL particles to the monolayer surface. In parentheses, 7C9/1D1 ratio (Table II) of 1.3 for monolayers suggests the preferential shift toward apoE association. Thus the ECM has a high capacity to sequester apoE from exogenous lipoproteins without significant binding of the lipoprotein particle to the matrix surface. However, it cannot be ruled out that the ECM preferentially binds VLDL subclasses that are highly enriched in apoE.

**ApoE Binding Components of the Cytochalasin B-prepared ECM**—It is generally accepted that apoE binds to HSPG via its heparin binding domains (34). The exogenous addition of heparin or suramin would be expected to competitively displace apoE from HSPG of the ECM. The endogenous apoE remaining after incubations was determined by measuring the specific binding of 125I-7C9 to the matrix. Incubation of the matrix with heparin at 37 °C had no significant effect on the endogenous apoE content (Table III). Suramin incubations at 37 °C and heparinase (3 units/ml, 37 °C) did not cause a release of endogenous apoE. Suramin incubations of 125I-7C9 antibody for 3 h. Unbound antibody was removed with three washes with PBS containing CaCl₂. The ECM was solubilized overnight in 0.5 N NaOH and then counted in a γ counter. ECM incubated with PBS-BSA, EMEM-BSA, or PBS bound 3177 ± 426, 2813 ± 120, and 3186 ± 314 cpm of 125I-7C9/well, respectively. Endogenous apoE experiments represent the mean and S.E. of three separate determinations.

The effect of the release conditions on the ability of 7C9 to recognize apoE was tested by passively adsorbing 0.5 μg of apoVLDL onto microroller wells as described under “Experimental Procedures” and incubating the immobilized samples with the release conditions. The wells were washed twice with PBS, blocked with PBS-BSA, and then incubated with 100,000 cpm/well of 125I-7C9. The microroller wells were then washed three times and counted. The counts for PBS-BSA controls were 8919 ± 380 cpm, and each condition was tested with six wells. Similar low levels of VLDL binding in ECM fractions were obtained using 125I-labeled VLDL (results not illustrated).

We also considered the ratios of the absolute changes in 7C9 and 1D1 immunoreactivity following incubations with VLDL. The 7C9/1D1 ratio (Table II) of 1.3 for monolayers suggests the binding of intact VLDL particles to the monolayer surface. In contrast, the 7C9/1D1 ratios for the Triton X-100 and cytochalasin B matrices were 32.1 and 13.7, respectively, indicating a preferential shift toward apoE association. Thus the ECM has a high capacity to sequester apoE from exogenous lipoproteins without significant binding of the lipoprotein particle to the matrix surface. However, it cannot be ruled out that the ECM preferentially binds VLDL subclasses that are highly enriched in apoE.
We have used apoE-poor ECM prepared by the Triton X-100 method. The apoE content of the ECM, as indicated by $^{125}$I-7C9 binding activity, increased the most when lipid-free apoVLDL was incubated with ECM. Less binding was observed when apoVLDL was preincubated with an equal amount (w:w) of PCV and less still when apoVLDL was preincubated with a 10-fold (µg:µg) amount of PCV. We then examined the effect of PCV or suramin on lipid-free apoVLDL that had been prebound to the Triton X-100 ECM. Incubation with 1 mg/ml PCV or 1 mg/ml suramin displaced approximately equal amounts of apoE (34 to 36%) from the ECM (Fig. 6). Sequential incubations with PCV and suramin displaced 53% of the lipid-poor apoE from the ECM.

**Endogenous ApoE of the Cell Surface and Cytochalasin B ECM Is Lipid Poor**—To test the effects of lipid complement on the immunoreactivity of anti-apoE monoclonal antibodies, we coated microtiter wells with either 0.5 µg of apoVLDL or 5 µg of VLDL and then incubated these immobilized ligands with increasing concentrations of Liposyn II, a solubilized triglyceride-rich emulsion, or POPC-cholate micelles. The immunoreactivity of antibodies reacting at three distinct epitopes was analyzed: 7C9 at the N terminus, 1D7 in the receptor binding domain, and 3H1 in the lipid binding domain. In wells that had not been coated with apoVLDL, only background counts were obtained, and there was no effect of increasing lipid (data not shown). Fig. 7 demonstrates that incubations of immobilized apoVLDL with Liposyn II or POPC induces a concentration-dependent increase in its immunoreactivity with 3H1. Maximal 3H1 immunoreactivity toward immobilized apoVLDL (3.8-fold) was achieved rapidly with 2.5–20 µg of Liposyn II (Fig. 7, top panel, solid symbols). Studies in which VLDL was adsorbed to the microtiter wells demonstrated maximal 3H1 immunoreactivity even in the absence of added lipids; the immunoreactivity was not significantly affected by further addition of Liposyn II (Fig. 7, top panel, hollow symbols). A more gradual increase in 3H1 immunoreactivity was achieved with POPC micelles (Fig. 7, bottom panel) with a maximal 4.3-fold increase observed at
suggest that much of the apoE on the ECM and cell surface determinations. 7C9. The data represent the means and S.E. of three separate determinations.

20–40 µg of POPC. The immunoreactivity of 1D7 (Fig. 7, top panel), a monoclonal antibody directed against residues 142–158 of apoE (LDL receptor and heparin binding region), also exhibited a 1.5-fold increase in binding with increasing Liposyn II. In contrast, the immunoreactivity of 7C9 was unaffected by the addition of Liposyn II (Fig. 7, top panel) or POPC cholate micelles (Fig. 7, bottom panel).

We next tested the effects of Liposyn II on the immunoreactivity of endogenous apoE of the cytochalasin B-derived ECM and cell surface with 3H1 and 7C9 (Fig. 8). At 4 °C, incubation with 1 mg/ml Liposyn II increased the immunoreactivity of 3H1 by 2-fold in cell monolayers and by 3.4-fold in matrix (Fig. 8, top left panel). 7C9 binding at 4 °C was unaffected by the presence of Liposyn II (Fig. 8, bottom left panel). Liposyn II incubations at 37 °C increased the immunoreactivity of 3H1 (top right panel) by about 2-fold in cell monolayers and 5-fold in ECM. Incubation of ECM with 1 mg/ml PCV at 37 °C also increased 3H1 immunoreactivity by 3.7-fold (data not shown). These results suggest that much of the endogenous apoE of ECM, and to a lesser extent the cell surface, exists in a lipid-poor form. In addition, incubations of cell monolayers with Liposyn II decreased the content of immunodetectable apoE as indicated by a 30% reduction in 7C9 binding activity (Fig. 8, bottom right panel), indicating either increased release or metabolism of cell surface apoE in the presence of Liposyn II. Incubation of the ECM with Liposyn II, however, did not result in a significant decrease in 7C9 binding activity although we observed earlier that incubation of the ECM with PCV at 37 °C did significantly reduce the 7C9 binding activity of this fraction by 30% (Table III). Table IV summarizes the Scatchard analysis of 7C9 and 3H1 binding to the cell surface in the presence and absence of Liposyn II treatment. Liposyn II induced no change in the affinity of binding for either antibody and had no effect on the number of available 7C9 epitopes. Liposyn II did, however, promote a significant 1.7-fold increase in the number of 3H1 epitopes on the cell surface (p < 0.005). Collectively, the data suggest that much of the apoE on the ECM and cell surface may exist as a lipid-poor oligomeric form. Treatment with Liposyn II could disassemble the oligomer, revealing additional 3H1 binding sites.

FIG. 6. Displacement of lipid-poor apoE from the ECM with PCV and suramin. Lipid-free apoVLDL (100 µg of protein/well) was incubated with Triton X-100-prepared ECM as described in the legend to Fig. 5. The ECM was washed twice with EMEM-BSA to remove free apoVLDL and then incubated at 37 °C with PBS-BSA (white bar) or PCV (1 mg/ml), suramin (0.5 mg/ml), or sequentially with PCV and suramin (shaded bars). apoE was determined by incubation with 125I-7C9. The data represent the means and S.E. of three separate determinations.

FIG. 7. Immunoreactivity of monoclonal 3H1, 1D7, and 7C9 to apoVLDL following incubation with Liposyn II and POPC micelles. ApoVLDL (0.5 µg in 100 µl, solid symbols) or human VLDL (5 µg in 100 µl, white symbols) in PBS was immobilized by passive adsorption to microtiter wells for 16 h at 4 °C. The wells were washed three times and then incubated with 100-µl volumes of PBS containing 0–40 µg of Liposyn II (top panel) or POPC micelles (bottom panel) for 2 h at 37 °C. The wells were then washed and blocked with PBS containing 1% fatty acid-free BSA. This was followed by incubation with radiolabeled 3H1, 1D7, or 7C9 (100,000 cpm/well) in PBS-BSA for 2 h at 25 °C, washing, and counting in a γ counter. The data represent the means and S.E. of three separate determinations.

**DISCUSSION**

A heparin/heparinase-releasable pool of apoE on the HepG2 cell surface has previously been described (16, 17). Cell surface apoE has also been characterized in rat hepatoma McA-RH7777 cells transfected with human apoE3 (18). These studies have not assessed cell surface apoE directly but rather measured increases in the levels of medium apoE after incubations with heparin, heparinase, or suramin. The specific binding of monoclonal 7C9 measures changes in cell surface apoE directly as indicated by the 30% decrease of cell surface apoE and the parallel increase in medium apoE following incubation with suramin (Fig. 1). We have used 7C9 binding to examine the endogenous apoE content and binding of apoE-containing lipoproteins to ECM prepared by three different methods. Preparation with cytochalasin B yields ECM with the highest endogenous apoE content (23.6% of the binding of cell monolayers). When compared with control incubations, the cytochalasin B method, in contrast to the protocols using Triton X-100 and EDTA, did not promote the dissociation of exogenously added apoE from the ECM. In terms of apoE content, we believe that the cytochalasin B-prepared material best represents the native HepG2 ECM. ECM prepared with Triton X-100 or EDTA demonstrates lower endogenous apoE levels (6...
and 3%, respectively, of cell monolayers). Regardless of the preparation method used, ECM material represents less than 1% of the total cellular protein. Despite this minor representation, the ECM can attain levels of apoE that represent about 60% of the HepG2 monolayer. This is evident after incubation with VLDL (Table I). In contrast, incubation of cytochalasin B-prepared ECM with EDTA, while removing one-third of the endogenous apoE content, results in a matrix preparation with 16% of the monolayer content. This difference might be explained by the varied nature of the starting materials. As cells separate from the ECM during the EDTA preparation protocol, cell surface molecules, for example HSPG, could pull endogenous apoE away from the ECM. This additional removal of apoE would not be expected when examining the effect of

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The association of apoE with the ECM appears to be heterogeneous. Suramin removes 37.4% and heparinase about 20% of the endogenous apoE of the ECM, suggesting attachment of a population via HSPG (Table III). We have found that incubations with heparin under various conditions (1–5 mg/ml, 4 and 37 °C) do not reproducibly affect the apoE content of the ECM. This varies from previous reports (16–18) and probably reflects differences in the interaction and association of apoE with the cell surface and the ECM. About one-third of the apoE associates with the ECM in a divalent cation-dependent manner as indicated by displacement with EDTA or EGTA. EGTA is a specific chelator of Ca2+ ions, suggesting that Ca2+-dependent interactions are involved in the binding of apoE to the ECM. In this regard, several components of the ECM have been shown to interact with extracellular proteins in a Ca2+-dependent manner. Fibulin-2 binding to fibronectin and nidogen requires Ca2+ (35) as does SPARC/BM-40 binding to collagen IV (36, 37). Of particular interest is a recent report describing an interaction between apoE and laminin (38). HepG2 cells express and secrete the laminin B chain (39), and laminin interacts in a calcium-dependent manner with several components of the ECM including the dystrophin-glycoprotein complex (40), calreticulin (41), BM 90/fibulin (42), and HSPG (43, 44). Our results therefore could be explained by a Ca2+-dependent conformation of matrix elements, including laminin and HSPG, which controls apoE binding.

The data from several experiments indicate that apoE also associates with the ECM in a lipid-dependent manner. PCV efficiently displaced about 30% of the endogenous apoE from the ECM either by itself (Table III) or following incubations with suramin or EGTA (Fig. 3). The additive nature of these sequential incubations suggests that the lipid-releasable pool and the suramin/EGTA-releasable pool associate differently with components of the ECM. A similar conclusion resulted from the studies of Getz et al. (45) who described additive release of apoE from the HepG2 cell surface by heparin and phospholipid vesicles. Surprisingly, Liposyn II, though displacing about 30% of the cell surface apoE from HepG2 monolayers, was ineffective at displacing apoE from the ECM (Fig. 8). As with the heparin data, this further delineates the differences between the association of apoE with components of the cell surface and ECM. In other experiments, lipid-free apoE derived from apoVLDL bound most efficiently to the ECM, whereas increasing the phospholipid:protein ratio to 1:1 or 10:1 (w:w) decreased apoE association by 45.8 and 58.6%, respectively. Approximately 35% of the lipid-free apoE could be displaced from the ECM by incubation with PCV or suramin; sequential incubations removed 53.3%. This data indicates that exogenously applied and lipid-poor apoE associates equally well with the suramin/EGTA- and lipid-releasable pools.

In many studies, we have found that the effects of EDTA, while always reducing the apoE content of the matrix, were variable. Preparation of ECM with EDTA, for example, results in matrix with a very low apoE content (3% of the monolayer content, Table I). In contrast, incubation of cytochalasin B-prepared ECM with EDTA, while removing one-third of the endogenous apoE content, results in a matrix preparation with 16% of the monolayer content. This difference might be explained by the varied nature of the starting materials. As cells separate from the ECM during the EDTA preparation protocol, cell surface molecules, for example HSPG, could pull endogenous apoE away from the ECM. This additional removal of apoE would not be expected when examining the effect of

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EDTA on the cytochalasin B prepared-ECM. In other studies in which the apoE content of the Triton X-100-prepared ECM had been increased by incubation with conditioned medium, we observed that EDTA could remove about 70% of the apoE content of the matrix (Fig. 2). Exogenously applied lipoproteins may associate with the isolated matrix in a different manner and may be more readily released by EDTA than endogenous apoE of the ECM. Based on our 3H1 immunoreactivity studies, we would expect lipoproteins of conditioned medium to have a greater proportion of associated lipid than apoE of the ECM and a decreased affinity for the ECM. This is particularly evident when considering the varied displacements of endogenous apoE and human VLDL from the ECM with suramin. We have found that suramin can displace 51.8 ± 8.0% of human VLDL from the Triton X-100-prepared ECM. This contrasts with the 37.3% displacement of endogenous apoE from the cytochalasin B-prepared ECM with suramin. Thus, lipidated lipoproteins may be more readily released from the matrix by suramin, EDTA, and possibly other treatments.

ApoE may be secreted from HepG2 cells (46, 47) or primary rat adult hepatocytes (48) in association with large, apoB-containing lipoproteins when cellular lipogenesis and VLDL production are stimulated or as relatively lipid-poor α-migrating particles (47). It has also been reported that 25-hydroxycholesterol promotes a significant redistribution of apoE from HDL to a density greater than 1.21 g/ml in infranatant (49) in HepG2 cells. This dense apoE fraction was composed of 85% of weight protein and 15% of weight of free cholesterol and phospholipid, and several methods of analysis demonstrated that its appearance was not an artifact of ultracentrifugation. To examine the lipid content of apoE of the ECM and cell surface, we have used the monoclonal 3H1 antibody. The immunoreactivity of 3H1, which recognizes an epitope in the C-terminal domain of apoE, increases in a concentration-dependent manner with the addition of Liposyn II or POPC micelles. The C-terminal domain of apoE contains amphipathic α-helices, binds avidly to lipoproteins (50, 51), and is responsible for the formation of the stable tetramer of apoE in aqueous solution (51, 52). Lipid association would be expected to alter both the conformation of the C-terminal region and the immunoreactivity of 3H1. Scatchard analysis of cell surface apoE demonstrated that Liposyn II alters the number of available antigen binding sites rather than changing the affinity of 3H1 for apoE. In this regard, incubation of immunoaffinity-purified apoVLDL with Liposyn II or POPC cholates micelles induced an approximate 4-fold increase in the immunoreactivity of 3H1, suggesting tetramer to monomer conversion. The immunoreactivity of 3H1 to endogenous apoE of the ECM also increased 3.2- and 5-fold with Liposyn II incubation at 4 and 37 °C, respectively. Incubations of HepG2 monolayers with Liposyn II at 4 and 37 °C increased 3H1 immunoreactivity by 2-fold. Interpretation of the effects of Liposyn II on the apoE of monolayers is complicated by several factors, including membrane turnover and metabolism at 37 °C. This is apparent in the TCA analysis where a substantial decrease in cell surface apoE is observed in the presence of Liposyn II. In addition, a substantial proportion of the Liposyn II effect in monolayers is probably due to the presence of apoE of the ECM. Regardless, the data suggests that apoE of the ECM exists in a lipid-poor form.

The function of endogenous ECM apoE may prove as complex or heterogeneous as the mode of apoE association with the ECM. Our results indicate that association of lipoprotein particles with the lipid-poor apoE on the ECM would decrease the affinity of apoE for its binding sites on the ECM and result in the subsequent release of the lipoprotein and its newly acquired apoE. As with the secretion-capture hypothesis of Ji et al. (18, 20) the net result would be an apoE-enriched lipoprotein particle with an increased predisposition for binding to the LDL receptor and the LRP. A similar mechanism could be used to enhance the apoE content of newly secreted lipoprotein particles. Hepatocytes secrete HDL that contains either or both apoA-I and apoE (53, 54). Although apoA-I is the major apolipoprotein of plasma HDL, newly secreted HDL has been shown to range from particles containing mostly apoA-I to others containing mostly apoE (55). The present results indicate that lipid-poor apoE of the ECM and cell surface can be transferred to the newly secreted lipoproteins as they emerge into the space of Disse and as required for lipid transport. In this regard, we have found that incubations of HepG2 cells with suramin decreases both cell surface apoE and the apoE content of newly secreted apoB-containing lipoproteins while having no effect on the secretion of apoE from intracellular stores. In macrophage, lipid-poor apoE of the ECM and cell surface might also participate in the reverse cholesterol transport (56, 57). Incubations of a transformed macrophage cell line constitutively expressing human apoE at 4 °C with lactoferrin or PCV promoted the release of apoE to the extracellular medium (58), suggesting, like HepG2 and McA7777 cells, that macrophage maintain apoE at the cell surface. Binding of lipoproteins to cell surface apoE would decrease the affinity of the apoE-lipid complex for the ECM/cell surface, which would then dissociate and move into the circulation.

In conclusion, we have shown that apoE is bound to the ECM of HepG2 cells via charge interactions that depend on the presence of Ca2+, either directly for binding to the ECM or indirectly to maintain the appropriate ECM structure. The ECM apoE is mostly lipid-poor and forms oligomers that are releasable by lipids and lipoproteins. This protein may serve to arm lipoproteins with apoE that is used for both lipid transport and lipoprotein uptake.

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