ApoE of the HepG2 Cell Surface Includes a Major Pool Associated with Chondroitin Sulfate Proteoglycans†

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Received August 17, 1998; Revised Manuscript Received October 5, 1998

ABSTRACT: We have investigated the association of apolipoprotein E (apoE) with the HepG2 cell surface (i.e. plasma membrane and extracellular matrix) using domain specific monoclonal antibodies against apoE. Growth in β-D-xyloside decreased the incorporation of ^{35}S into glycosaminoglycans by 31% and cell surface apoE by 45% with a concomitant increase in apoE secretion (4.3-fold), underlining the importance of glycosaminoglycan association of apoE. Heparinase (3−10 U/mL) or heparin (1 mg/mL) decreased apoE by 25 and 30.5%, respectively, which suggests that some apoE is associated with cell surface heparan sulfate proteoglycans. Chondroitinase ABC (1.5 U/mL) reduced cell surface apoE by 40%, indicating that a major pool of apoE is associated with chondroitin sulfate proteoglycans. Further enzymatic and displacement analysis suggested that cell surface apoE associates specifically with GAGs containing chondroitin-4-sulfates. 3H1, a monoclonal antibody that recognizes an epitope within the lipid-binding C-terminal domain of apoE, decreased binding of apoE to chondroitin sulfate proteoglycans in solid-phase assays by 77% and to heparan sulfate proteoglycans by 46%, suggesting that this region is of increased importance for binding to chondroitin sulfate proteoglycans. Previous studies with 3H1 demonstrated that apoE of the extracellular matrix is lipid-poor (Burgess, J. W., Gould, D. R., and Marcel, Y. L. (1998) *J. Biol. Chem. 273*, 5645−5654), but we show here that apoE on the remaining cell surface is lipid-associated. In summary, lipidated apoE associates with the HepG2 plasma membrane through interactions with chondroitin-4-sulfate containing GAGs and, to a lesser extent, HSPG.

Apolipoprotein E (apoE)[1] is a 33 kDa exchangeable apolipoprotein that plays a central role in the intravascular metabolism of cholesterol and triglycerides. In plasma it is one of the major constituents of cholesterol ester-rich lipoproteins (chylomicrons, VLDL, their remnant particles, and HDL; for review, see reference *1*). Lipoproteins containing apoE bind to the LDL receptor, the low-density lipoprotein receptor-related protein (LRP), and heparan sulfate proteoglycans (HSPG; *2*, *3*). Circulating apoE is mostly of hepatic origin (*1*), but a significant proportion of the apoE secreted by hepatocytes into the Space of Disse is believed to be transferred directly to the surface of chylomicron and VLDL remnants, thereby enhancing their ability to bind to HSPG and lipoprotein receptors of the hepatocyte cell surface (secretion-capture model) (*4*−*6*). The importance of apoE in remnant metabolism has been demonstrated by dysfunctional mutations of apoE, which lead to elevated plasma cholesterol levels and increased risk of heart disease (*7*−*9*).

Histochemical analysis has demonstrated that apoE is also associated with the basolateral surfaces of hepatic parenchymal cells (*10*) and adrenocortical parenchymal cells (*11*). Biochemical studies which have involved incubation of HepG2 cells (*12*, *13*) or McA7777 cells transfected with human apoE3 (*5*) with heparin or heparinase have found an increased content of apoE in the surrounding medium, suggesting attachment with the cell surface through HSPG. We have recently characterized the association of apoE with the extracellular matrix (ECM) of HepG2 cells by assessing the specific binding of radiolabeled monoclonal anti-apoE antibodies to this cell fraction (*14*). This methodology has allowed us to identify apoE-matrix interactions that are dependent on HSPG, calcium, and lipid. ApoE of the ECM represents about 25% of the immune reactive apoE of the cell surface, suggesting that a substantial pool also exists on the plasma membrane of HepG2 cells (*14*).

Much evidence indirectly supports the notion that hepatic cell surface apoE plays an important role in lipoprotein processing, binding, and uptake. Immunohistochemistry of hepatocytes from transgenic mice expressing apoE demonstrated localization of apoE at the basolateral surface and a marked reduction in this pool following a bolus injection of chylomicron remnants. This disappearance was associated with a coincident increase of apoE associated with intracellular vesicles (*6*). The studies of Linton et al. (*15*) further suggest that apoE of hepatic origin, but not free in the Space of Disse, is important for remnant metabolism in mice lacking a functional LDL receptor. These studies therefore implicate cell surface apoE in remnant metabolism. To understand how cell surface apoE contributes to lipoprotein metabolism, we have undertaken the characterization of the

† This research was supported by a grant from the Heart and Stroke Foundation of Ontario.

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[1] Abbreviations: apoE, apolipoprotein E.; BSA, bovine serum albumin; CSPG, chondroitin sulfate proteoglycan; DSPG, dermatan sulfate proteoglycan, ECM, extracellular matrix; EMEM, Eagle's minimum essential medium; DMPC, dimyristoyl phosphatidylcholine; GAGs, glycosaminoglycans; HSPG, heparan sulfate proteoglycans.

10.1021/bi9819778 CCC: $18.00
Published on Web 12/19/1998

membrane-associated molecules that are responsible for apoE cell surface presentation and how they may control apoE turnover and function. We have found that the majority of apoE associates with the cell surface through interaction with chondroitin sulfate proteoglycans and that apoE present on the plasma membrane is lipidated.

MATERIALS AND METHODS

Materials. $Na^{125}I$ was obtained from Amersham (Oakville, ON). Iodobeads were from Pierce (Rockford, IL). Heparin from pork intestinal mucosa, suramin, heparinase type 1, chondroitinase ABC, chondroitin sulfates A−C, egg phosphatidylcholine, (4-methylumbelliferyl)-β-D-xyloside, and purified HSPG were obtained from Sigma (St. Louis, MO). Protease-free chondroitinase ABC, produced by Seikagaku Kogyo Co. (Tokyo, Japan), and $[^{35}S]Na_2SO_4$ was obtained from ICN (Mississauga, ON). 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 from Abbott Laboratories (Montreal, Quebec). Purified chondroitin sulfate proteoglycans (CSPG) and dermatan sulfate proteoglycans (DSPG) were obtained from Collaborative Biomedical Products (Toronto, Canada). Tissue culture reagents were obtained from Sigma and GIBCO (Burlington, ON). HepG2 cells were obtained from the ATCC.

Antibodies. The monoclonal antibodies (mAbs) against apoE used in these studies have been described earlier (*14*, *16*, *17*) and include 7C9 and 6C5 which recognize residues 1−15 of the N-terminus, 1D7 and 2E8 which recognize the region encompassed by residues 142−158, and 3H1 which recognizes residues 220−272 of the C-terminus. Iodination of antibodies was performed by the iodobead method (Pierce), and specific activities between 8 and 10.6 uCi/ug were obtained.

Cell Culture and Characterization of Cell Surface apoE. HepG2 cells were cultured as described previously (*14*). To label cellular glycosaminoglycans, $Na_2{}^{35}SO_4$ (10 μCi/mL) was added to the cultures at the time of seeding and again on day 3 after seeding. Experiments were performed on day 4 after seeding. In some studies HepG2 cells were incubated for 4 days with 2.5 mM (4-methylumbelliferyl)-β-D-xyloside with a change of media and drug on the third day of treatment.

The apoE content of cell monolayers was determined using radiolabeled mAb 7C9 or other antibodies reacting also with the N-terminus (not illustrated). Cells were prechilled 15 min on ice and then washed 3 times in ice-cold EMEM containing 2 mg/mL fatty acid free BSA (EMEM-BSA). The cells were then incubated with various reagents, including heparin, suramin, and chondroitin sulfates as described in the figure legends. Enzymatic treatments were conducted at 37 °C for 2 h. Iodinated 7C9 (2×10^6 cpm; representing 300 ng of 7C9 protein) was added to each well containing approximately 0.5 mg of cell protein. This amount of 7C9 was sufficient to fully saturate the HepG2 cell surface (Figure 1). To determine nonspecific binding, a 100-fold excess of cold mAb was added to replicate wells. The mAbs were incubated on ice for 2−4 h, and then cells were washed 3 times in cold PBS containing 1.7 mM $CaCl_2$ and solubilized overnight with 1 mL of 0.5 M NaOH. Radioactivity was measured in a γ counter, and the results for antibody binding are expressed as the mean specific binding per milligram of cell protein.

Effect of Lipids on apoE Immunoreactivity at the Surface of Suspension Cells. HepG2 cells were grown to confluency in T-175 cm^2 cell culture flasks. The medium was changed the day before the determination of cell surface apoE. The cells were released from the substratum by incubation with 3 mM EDTA at 37 °C as described by Owensby et al. (*18*), collected, and chilled in 50 mL conical centrifuge tubes. The cells were washed twice at 4 °C with EMEM-BSA (1300 rpm for 5 min/PR6000 Beckman centrifuge), suspended in 25 mL of EMEM-BSA, and then divided into 24−1.5 mL conical Eppendorf tubes with screw caps (1 mL of cell suspension per tube). Liposyn II was added to half of the tubes (final concentration of 1 mg/mL), while the remaining tubes received an equivalent volume of EMEM-BSA. The cells were incubated at 4 °C for 1 h on a rotating mixer set on slow speed and then washed twice in PBS-BSA. Radiolabeled 3H1 or 7C9 (2×10^6 cpm; 300 ng of IgG) was added to triplicate tubes. Nonspecific binding, also in triplicate, was determined with a 100-fold excess of unlabeled antibody. The antibody incubations were performed at 4 °C on a rotating mixer for 2 h. The cells were then washed 3 times with PBS−Ca^{2+}, solubilized overnight in 0.5 N NaOH, and then counted in a γ counter. Results are expressed as the mean specific binding per milligram of cell protein.

Determination of Media Apolipoproteins by Radioimmunoassay. A sandwich assay, using 6C5 as the capture antibody, and iodinated 3H1 as the detection antibody, was used to measure apoE secreted into the medium of HepG2 cells. Pure 6C5 IgG at 1 ug/well was coated onto microtiter wells in PBS and incubated at 4 °C for 18 h. The wells were then blocked with 3% BSA in PBS for 1 h, washed 3 times in PBS, and incubated with standards or test samples in the presence of 1% BSA for 2 h. Wells were then washed 3 times in PBS, containing 0.1% β-octylglucylpyranoside, and incubated with iodinated 3H1 (100 000 cpm/well) in PBS containing 1% BSA and 0.1% β-octylglucylpyranoside for 1 h. After three washes with PBS containing 0.1% β-octylglucylpyranoside, the wells were counted in a γ counter. A stock of conditioned medium with a known concentration of apoE (quantified using a pure apoE standard curve) was used as the standard in regular assays. Lipidation of apoE has no effect on the affinity of 3H1 for apoE but does increases the number of sites recognized by this antibody as a result of lipid-induced unfolding of the apoE tetramer (*14*). Inclusion of 0.1% β-octylglucylpyranoside during incubations with 3H1 eliminates the effects of lipid on 3H1 reactivity (data not shown), possibly because it also promotes unfolding of the apoE tetramer, allowing the determination of total apoE (i.e. lipid poor + lipid replete) in conditioned medium from HepG2 cells.

ApoE Binding to Immobilized GAGs. Purified HSPG from Engelbroth−Holm−Swarm mouse sarcoma, CSPG, and DSPG from bovine aorta (1 μg in 100 μL of PBS) were immobilized by passive adsorption to 96 well microtiter plates (Immobilon 2; Dynatech Laboratories) for 16 h at 4 °C. Unbound material was removed with three washes of PBS, and the wells were then blocked with 250 μL of 3% BSA in PBS for 1 h at room temperature. ApoVLDL (2.5 μg/100 μL) that had been preincubated for 1 h at 20 °C with monoclonal antibodies (1.0 μg/μg apoVLDL) against apoE

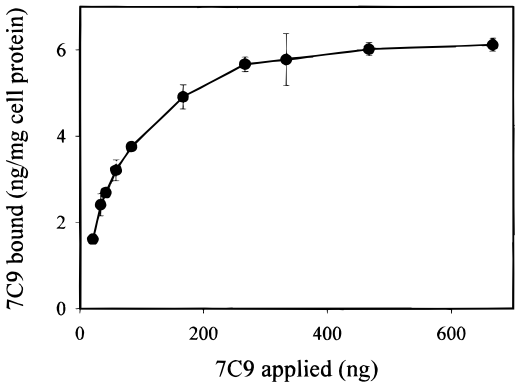

FIGURE 1: Optimizing the binding of 7C9 with the HepG2 cell surface. Confluent HepG2 cells were washed twice with ice-cold PBS−BSA and then incubated with increasing amounts of ^{125}I-7C9 (12000 cpm/ng of 7C9 protein) for 2 h at 4 °C as described on the *x*-axis. The cells were then washed three times with PBS containing 1.7 mM $CaCl_2$, solubilized overnight in 0.5 N NaOH, and counted. Complete saturation represents the binding of 1.8−2 nmol of 7C9 IgG protein/0.5 mg of cell protein. All data points are the mean and SE of three separate determinations.

(1D7, 2E8, and 3H1) or apolipoprotein A1 (4H1) was added to the wells and incubated for 2 h at 37 °C. In some experiments apoVLDL, which had been incubated with control antiserum (4H1), was further incubated in the absence and presence of DMPC vesicles (3 μg of lipid/μg of protein) at 37 °C for 2 h prior to addition to the immobilized glycosaminoglycans. The wells were washed 3 times with PBS and then incubated with ^{125}I-7C9 (100 000 cpm/well) for 2 h at 20 °C. The wells were washed 3 times with PBS and counted.

Miscellaneous Procedures. VLDL was prepared from the plasma of normolipidemic subjects by density gradient ultracentrifugation as described by Rall et al. (*19*). ApoVLDL was prepared from VLDL as described (*19*). Briefly, VLDL was dialyzed against 0.01% EDTA, pH 7.4, and lyophilized. Lipids were then removed at 4 °C by extraction with chloroform:methanol (2:1, v/v), and the material was then washed with cold methanol, dissolved in 6 M guanidine, 0.1 M Tris, pH 7.4, 0.01% EDTA and 1% 2-mercaptoethanol, and solubilized overnight at room temperature. The insoluble material containing apolipoprotein B was then removed by centrifugation, and then supernatant was dialyzed against three changes of 100 volumes of 0.1 M NH_4HCO_3 containing 0.1% 2-mercaptoethanol. ApoVLDL containing mostly apoE and apolipoprotein C was then aliquotted and stored at −70 °C. Cell protein was determined by the method of Lowry et al. (*20*) with BSA as standard.

RESULTS

Effect of (4-Methylumbelliferyl)-β-D-xyloside on Cell Surface apoE. β-D-xyloside analogues substitute for xylose in the initiation of GAG synthesis on the proteoglycan core proteins. This results in a shift toward the synthesis and secretion of free GAG, and a reduction in GAG incorporation into cellular proteoglycans (*21, 22*). HepG2 cells were incubated for 4 days with 2.5 mM (4-methylumbelliferyl)-β-D-xyloside with a change of media and drug on the third day of treatment. Microscopic examination of cells grown with this concentration of xyloside demonstrated no apparent effect on cell viability or cell number (data not shown) in agreement with the findings of Lilly-Staudermann et al. (*12*).

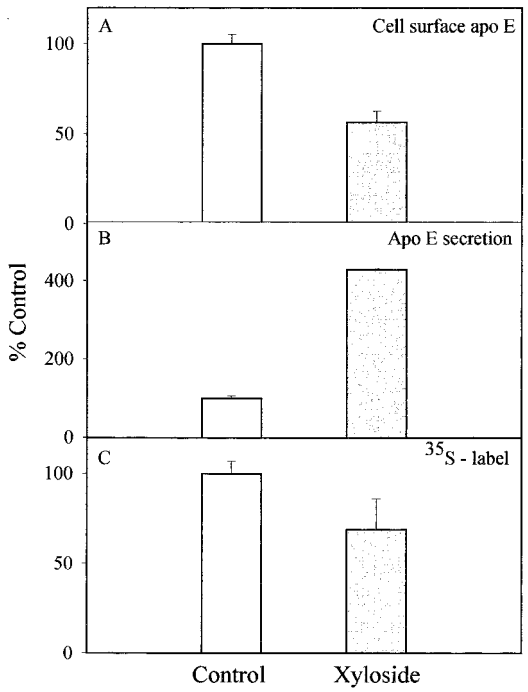

FIGURE 2: Effects of (4-methylumbelliferyl)-β-D-xyloside on cell surface apoE, apoE secretion, and glycosaminoglycan synthesis. HepG2 cells were grown to confluency in complete medium containing 2.5 mM (4-methylumbelliferyl)-β-D-xyloside. On the day prior to the experiment, fresh growth medium containing $Na_2{}^{35}SO_4$ (10 μCi/mL) and xyloside was added. The conditioned medium was assayed by sandwich RIA for secreted apoE, and cell surface apoE was determined after incubation with ^{125}I-7C9 as described in Experimental Procedures. ^{35}S counts were determined from NaOH solubilized cells using a scintillation counter. All data points are the means and SE of three separate determinations. In control, non-xyloside-treated cells ^{125}I-7C9 bound was 66 500 ± 3600 cpm/mg of cell protein, apoE secretion was 38 ± 1.2 μg/mg of cell protein in 24 h, and incorporation of ^{35}S counts were 512 111 ± 61 437 cpm/mg of protein.

Trypan blue exclusion studies demonstrated similar high cell viability (>98%) in both xyloside and untreated cells (data not shown). Growth in xyloside decreased cell surface apoE by 45% (Figure 2A) and cell-associated ^{35}S label by 31 ± 17% when the cellular GAGs had been metabolically labeled with [^{35}S]Na_2S0_4 (Figure 2C). Xyloside treatment was also associated with a 4.3-fold increase in apoE secretion (Figure 2B), indicating that the reductions in cell surface apoE were the result of reduced levels of surface GAGs rather than reduced secretion of apoE.

Enzymatic Release of apoE from the HepG2 Cell Surface. Several reports have described increases in the medium apoE following heparinase incubations of HepG2 (*12*, *13*) cells or McA-7777 cells transfected with human apoE (*5*). While these indirect methods have convincingly established an association of apoE with cell surface HSPG, they have not allowed quantification of apoE at the cell surface and have not ruled out other possible means by which apoE associates with the cell surface. Consequently, we have incubated ^{35}S−labeled HepG2 cells with heparinase or chondroitinases to release cell surface GAGs and then added ^{125}I-7C9 to determine changes in cell associated apoE. Figure 3 demonstrates that heparinase (3 U/mL) decreased the subsequent binding of ^{125}I-7C9 (upper panel) and cell associated ^{35}S (lower panel) by about 25% after 2 h incubations at 37 °C. Increasing the amount of heparinase to 10 U/mL did not

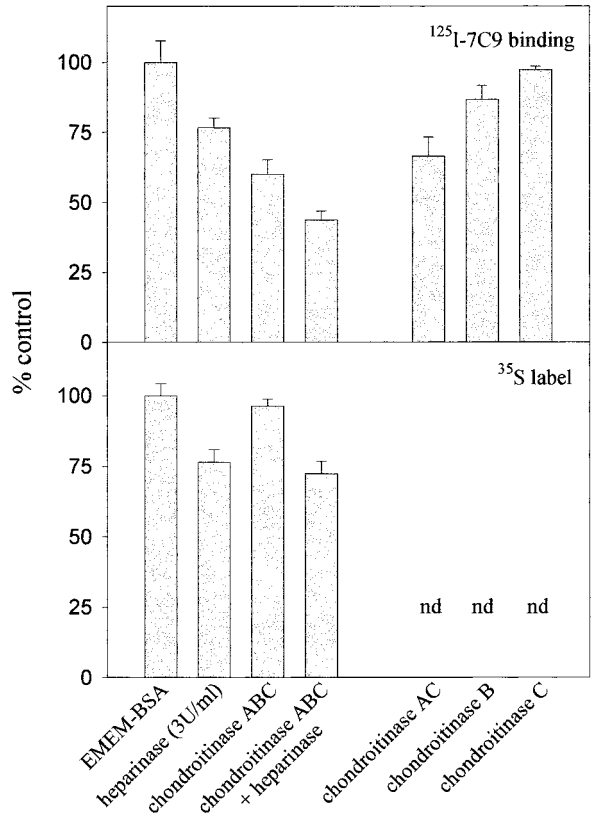

FIGURE 3: Enzymatic analysis of cell surface apoE. HepG2 cells were grown in the presence of $Na_2{}^{35}SO_4$ (10 μCi/mL) to label cellular GAGs. The cells were washed three times at 4 °C with EMEM-BSA. Heparinase (3 or 10 U/mL), chondroitinase ABC (1.5 U/mL), chondroitinase AC (1.5 U/mL), chondroitinase B (1.5 U/ml) or chondroitinase C (1.5 U/mL) was added and the incubations continued at 37 °C for 2 h. Cell surface apoE (upper panel) and ^{35}S-label (lower panel) were determined as described in the legend to Figure 2 and in Experimental Procedures. All data points are the means and SE of three separate determinations.

result in further decreases in 7C9 binding or the ^{35}S-label-indicating that saturating amounts of enzyme had been used. Most surprising were incubations with chondroitinase ABC which releases chondroitin-4-sulfate, chondroitin-6-sulfate, and dermatan sulfate from GAG chains. Chondroitinase ABC from Sigma (C 3667; 1.5 U/mL; 37 °C) decreased subsequent 7C9 binding to between 55.6 and 64.7% of control values in three separate experiments (average = 60.1 ± 5.1). During these incubations, cell-associated ^{35}S decreased by only 3.7% (Figure 3, lower panel) which likely reflects both the low sulfation (*23*) and content (*24*) of CSPG versus HSPG in cells of hepatic origin. A highly purified and protease-free chondroitinase ABC from Seikagaku Kogyo Co. similarly decreased 7C9 binding to 61 ± 5.6% of control values. Sequential incubations with chondroitinase ABC (1.5 U/mL) and heparinase (3 U/mL) decreased cell surface apoE to 43.7% of control values. Chondroitinase AC (1.5 U/mL) was almost as effective as chondroitinase ABC decreasing cell surface 7C9 binding to 66.5 ± 11% of control values (Figure 3 upper panel). Chondroitinase B was less effective reducing 7C9 binding to 86.7 ± 5.0% of control values. Chondroitinase C was without effect on cell surface apoE.

Effect of Incubations with Heparin, Suramin, and Chondroitin Sulfates on Cell Surface apoE. Incubation with 10 μg of heparin/mL at 37 °C decreased cell surface apoE by 25.3% (Table 1). Increasing the heparin concentration to 1 mg/mL decreased cell surface apoE by 30.5%; further increases in heparin concentration to 5 mg/mL released no more apoE from the cell surface (data not shown). Incubation with suramin was not effective at 10 μg/mL but very efficiently decreased cell surface apoE by 52% at concentrations of 1 mg/mL. Since chondroitinase ABC effectively decreased cell surface apoE (Figure 3), we also examined the effects of chondroitin sulfates on cell surface apoE. Chondroitin sulfates A and B (dermatan sulfate) were not effective at concentrations of 10 μg/mL; however, increasing the concentration to 1 mg/mL resulted in significant reductions of cell surface apoE; by 23.6% for chondroitin sulfate A ($p < 0.01$) and 15.3% for chondroitin sulfate B ($p < 0.1$). In contrast, chondroitin sulfate C was not effective at releasing cell surface apoE at either 10 μg/mL or 1 mg/mL. When incubations were performed at 4 °C, suramin (0.5 mg/mL) decreased cell surface apoE by 20.8% (data not shown). Incubations with heparin or the chondroitin sulfates at 4 °C had no effect on cell surface apoE (data not shown) possibly due to alterations in membrane dynamics or decreased rates of dissociation of apoE from GAGs at this reduced temperature.

Table 1: Effect of Heparin, Suramin, and Chondroitin Sulfates on Cell Surface apoE[a]

compound	conditions	^{125}I-7C9 bound, cpm × 10^{-3}/mg of protein	% removed
EMEM-BSA		72.2 ± 7.0	0
heparin	10 μg/mL	53.9 ± 2.3	25.3[b]
	1 mg/mL	50.2 ± 4.4	30.5[b]
suramin	10 μg/mL	73.6 ± 3.8	−1.9
	1 mg/mL	34.7 ± 1.7	52[b]
chondroitin sulfate A	10 μg/mL	72.8 ± 3.3	0
	1 mg/mL	55.2 ± 4.4	23.6[b]
chondroitin sulfate B	10 μg/mL	74.7 ± 4.1	0
	1 mg/mL	61.1 ± 6.9	15.3[c]
chondroitin sulfate C	10 μg/mL	78.2 ± 1.7	−8.3
	1 mg/mL	74.6 ± 3.0	−3.3

[a] Confluent HepG2 cells were washed three times at 4 °C with EMEM-BSA. Incubations with the indicated agents, all suspended in EMEM-BSA, were conducted at 37 °C for 1 h. The cells were then washed twice with PBS−BSA and then ^{125}I-7C9 binding was determined as described in the legend to Figure 1 and in Experimental Procedures. All data are the mean and SE of three separate determinations. [b] $p < 0.01$. [c] $p < 0.10$.

Changes in the 7C9 binding activity could be explained by changes in the levels of apoE at the cell surface or reduced binding of 7C9 due to conformational changes in apoE brought about by the incubations. We have previously shown that incubations of immobilized apoVLDL with heparin or suramin at 37 °C did not affect the subsequent binding of 7C9 to apoE (*14*). We have also found that incubations of immobilized apoVLDL with the chondroitin sulfates exert no effect on the binding of 7C9 to apoE (data not shown). Therefore decreases in 7C9 binding following incubations with heparin, suramin, or chondroitin sulfates A and B represent changes in the level of cell surface apoE.

ApoE−GAG Interactions. The Involvement of GAG Sulfation and Domains of apoE. The interaction of apoE with heparin involves arginyl and lysyl groups between residues 142 and 147 of the receptor binding region and between residues 202 and 243 of the lipid-binding region of apoE (*25*, *26*). It is believed that these positively charged residues facilitate an ionic interaction with the negatively charged

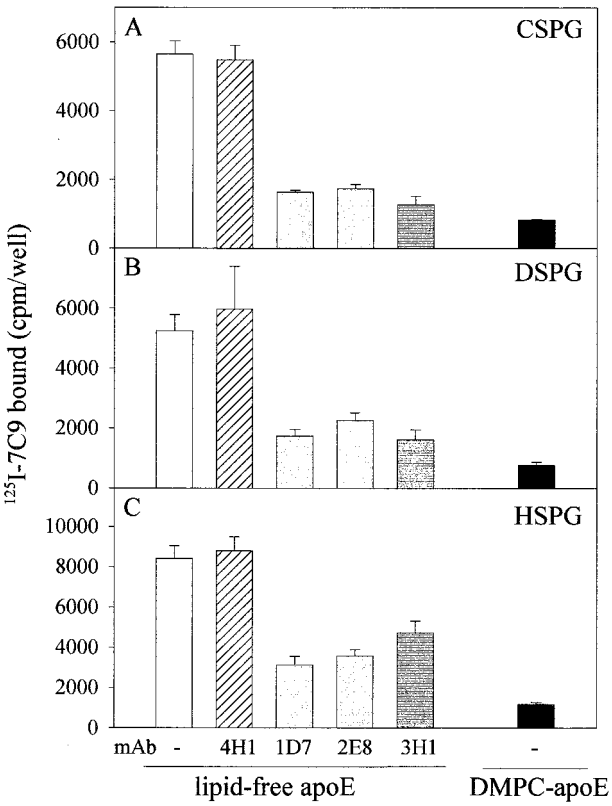

FIGURE 4: Effects of anti-apoE antibodies on the binding of lipid-free apoE to immobilized GAGs. ApoVLDL was preincubated for 1 h at 20 °C with PBS or monoclonal antibodies (0.6 μg/μg of apoVLDL) which recognize epitopes within the receptor binding region (1D7 and 2E8) or carboxy terminal tail (3H1) of apoE or against apolipoprotein A1 (4H1). The apoVLDL was incubated for 1 h at 37 °C in the absence or presence of DMPC vesicles (3 μg of lipid/μg of protein), and then added (2.5 μg/100 μL) to immobilized CSPG (A), DSPG (B) or HSPG (C) in 96 remova-well plates and incubated for 2 h at 37 °C. The wells were then washed 3 times with PBS containing 1% fatty-acid-free BSA and incubated with ^{125}I-7C9 (100 000 cpm/well) for 2 h at 20 °C. The wells were then washed 3 times with PBS and then counted in a γ counter. Control wells lacking immobilized glycosaminoglycans bound negligible amounts of ^{125}I-7C9 (212 ± 37 cpm) following incubation with apoVLDL.

sulfate and carboxylate groups present in the carbohydrate structure of heparin (*25*, *26*). To investigate the domains of apoE that interact with CSPG/DSPG, we preincubated apoVLDL with PBS or monoclonal antibodies against the receptor binding region (1D7 and 2E8) and C-terminus (3H1) of apoE or an irrelevant antigen (apolipoprotein A−I, epitope 4H1). These mixtures were then added to purified GAGs immobilized on microtiter wells. GAG-associated apoE was then detected with the addition of ^{125}I-7C9. In preliminary studies we found that pretreating immobilized apoVLDL with 1D7, 2E8, 3H1, or 4H1 did not affect the subsequent binding of ^{125}I-7C9 to apoE (data not shown). In addition, the association of apoE with heparin (*14*), chondroitin sulfate, or dermatan sulfate (data not shown) did not affect the binding of 7C9 to apoE. Therefore detection with this monoclonal antibody provides an accurate estimate of the amount of apoE associated with the tested GAGs. In agreement with previous reports (*28*), we found that preincubation with 1D7 and 2E8 markedly decreased binding of apoVLDL to HSPG to 35.5 and 40.6% of control values, respectively (Figure 4; panel C). The binding of apoVLDL

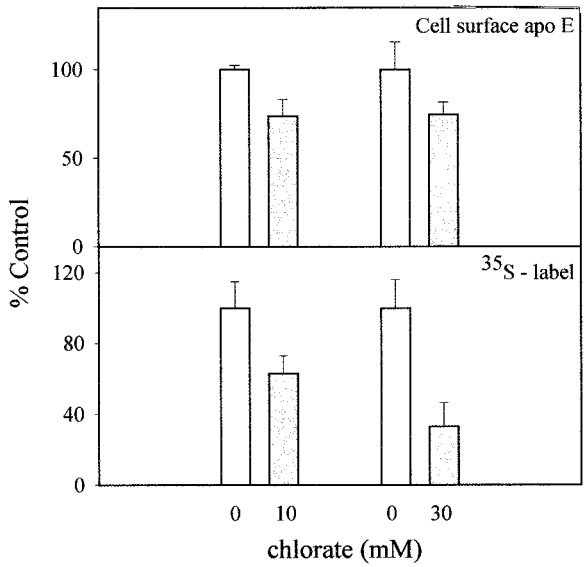

FIGURE 5: Effects of sodium chlorate on cell surface apoE and GAG sulfation. HepG2 cells were grown to confluence in the presence of $Na_2{}^{35}SO_4$ (10 μCi/mL) for 3 days. Fresh medium containing $Na_2{}^{35}SO_4$ and including either 10 or 30 mM sodium chlorate or sodium chloride was then added and the incubations continued for an additional 24 h. The cells were then washed with PBS−BSA and processed for 7C9 binding (upper panel) and ^{35}S determinations (lower panel) as described in the legend to Figure 2 and in Experimental Procedures. The data are means and SE of three separate determinations. In control cells ^{125}I-7C9 bound was 70 402 ± 3880 cpm/mg of cell protein and incorporation of ^{35}S counts were 568 613 ± 4952 cpm/mg of protein.

to immobilized CSPG and DSPG was similarly decreased by monoclonal antibodies 1D7 and 2E8 (Figure 4, panels A and B, respectively). In contrast 4H1, when compared to the PBS control, did not inhibit the binding of apoE to the immobilized GAGs. To determine the importance of the C-terminal lipid-binding domain of apoE in mediating association with GAGs, we incubated apoVLDL with monoclonal 3H1 before addition to the immobilized GAGs. Preincubation with 3H1 decreased the binding of lipid-free apoE to HSPG (46% inhibition compared to control) but exerted a much greater inhibition on the binding to CSPG (77%) and DSPG (73%). ApoE associated with DMPC vesicles bound poorly (13−15% versus controls) to all GAGs (Figure 4), possibly because the binding sites are masked or altered structurally by lipid binding. These results suggest that both the receptor-binding region and domains within the C-terminal tail of apoE are important for interaction with CSPG and DSPG in the absence of lipids.

To examine the importance of GAG sulfation in determining the levels of cell surface apoE, we grew cells in the presence of sodium chlorate. Chlorate is a potent inhibitor of sulfate adenyltransferase and consequently decreases sulfate incorporation into cellular GAGs (*27*, *28*). Control cells were grown in the presence of identical concentrations of sodium chloride. Growth in 10 or 30 mM sodium chlorate for 24 h decreased the ^{35}S-labeling of cells by 37 and 67%, respectively (Figure 5; lower panel). The chlorate concentrations employed in this study are within the range employed by others (*5*, *27*, *28*) and exerted no apparent effects on cell viability or cell number. Cell surface apoE at both chlorate concentrations was decreased by about 25% (upper panel), suggesting that the extent of GAG sulfation is important for

Table 2: Effect of Liposyn II on 3H1 and 7C9 Immunoreactivity of Cell Surface ApoE[a]

	Liposyn	3H1 reactivity (% control)	7C9 reactivity (% control)	3H1/7C9 ratio
monolayers	−	100 ± 4.9	100 ± 6.4	
	+	184 ±13.0	72.0 ± 6.0	2.6
suspension cells	−	100 ± 4.8	100 ± 7.5	
	+	132 ± 1.0	116 ± 17.3	1.1
ECM	−	100 ± 24.8	100 ± 8.4	
	+	510 ± 110	96.0 ± 7.0	5.3

[a] HepG2 monolayers, suspension cells, and extracellular matrix were incubated at 37 °C in the absence or presence of 1 mg/mL Liposyn II for 1 h. The cells or cell fractions were then washed at 4 °C twice with PBS−BSA and then incubated with ^{125}I-3H1 or ^{125}I-7C9 (2 × 106 cpm/well) for 3 h. The wells were then washed three times with ice cold PBS containing 1.7 mM $CaCl_2$ and then solubilized overnight with 0.5 N NaOH prior to counting in a γ counter. The binding of both antibodies to empty well controls was negligible (28 ± 16 and 44 ± 12 cpm/well for ^{125}I-7C9 in the absence and presence of the Liposyn II incubation; 38 ± 19 and 22 ± 16 cpm/well for ^{125}I-3H1). All data points were derived from three separate experiments.

the association of a minor pool of apoE with the cell surface. A larger pool associates with the cell surface in a manner that does not require full GAG sulfation. Growth of HepG2 cells in chlorate did not significantly alter secretion of apoE into the extracellular medium (data not shown).

Lipid Content of Cell Surface apoE. We have previously reported that the immunoreactivity of 3H1 for lipid-free apoE associated with the ECM dramatically increases following incubation with either Liposyn II, a solubilized triglyceride emulsion, or POPC micelles (*14*). In contrast Liposyn II incubations had no effect on the immunoreactivity of 7C9 toward apoE. To examine the lipid content of plasma membrane-associated apoE without contribution of apoE of the ECM, which we have shown to be lipid poor (*14*), we have used EDTA to release cells from the matrix. Cells released in this fashion remain greater than 98% viable by trypan blue exclusion (*18*; data not shown). Suspension cells, monolayers, and extracellular matrix were incubated at 37 °C with and without Liposyn II and then incubated at 4 °C with radiolabeled 3H1 or 7C9. Incubations with Liposyn II increased the 3H1 immunoreactivity of ECM by 5.1-fold (Table 2). The immunoreactivity of 3H1 toward apoE of cell monolayers and EDTA-released cells was much less affected by incubation with Liposyn II, increasing 1.84- and 1.32-fold, respectively. The immunoreactivity of 7C9 toward apoE is unaffected by lipid (*14*) and provides an indication of the relative levels of apoE in the cell fractions. The immunoreactivity of 7C9 decreased by 28% in monolayers following incubation with Liposyn II but was unchanged in ECM and suspension cells. This may reflect either increased release or metabolism of lipidated apoE in intact cells and the requirement for the ECM for this event to occur. The 3H1/7C9 ratio (Table 2) correlates change in the 3H1 immunoreactivity with Liposyn II relative to changes in the apoE content of the cell fractions. The 3H1/7C9 ratio for ECM and cell monolayers was 5.3 and 2.6, respectively, indicating that apoE of the ECM is mostly lipid-free whereas apoE of cell monolayers is relatively enriched in lipid. The presence of the ECM in the cell monolayers probably accounts for the elevated 3H1/7C9 ratio. In contrast the 3H1/7C9 ratio of 1.1 for suspension cells indicates that the apoE of the matrix-depleted surface is associated with lipid.

DISCUSSION

Previous examination of the specific binding of radiolabeled 7C9, a mAb against the N-terminal domain of apoE, to the ECM of HepG2 cells allowed us to demonstrate both heparinase- and lipid-releasable pools of apoE (*14*). In the current study we have extended our analysis to the entire HepG2 cell surface, which includes both the ECM and plasma membrane. Our immunometric assays provide a direct measurement of the steady-state levels of apoE associated with the cell surface and present in the medium. Previous studies have examined cell surface apoE by measuring increases in medium apoE following incubations of cells with heparin or heparinase (*5*, *12*, *13*). These indirect determinations are compromised by dilution of the released apoE with the secreted apoE pool. It is noteworthy that Ji et al. (*5*) found, using this protocol, increases of only 4 and 12% in the medium apoE following incubations of HepG2 cells with heparin and heparinase, respectively.

Our studies with (4-methylumbelliferyl)-β-D-xyloside indicate the importance of cell surface GAGs in the binding of apoE (Figure 2). Animal cells assemble GAG chains on β-D-xylosides to a greater extent than on endogenous core proteins, which promotes secretion of the primed material (*27*, *28*). HepG2 cells grown in the presence of 2.5 mM xyloside exhibit a 31% decrease in the amount of ^{35}S-label incorporated into cellular GAGs and a 45% decrease in cell surface apoE (Figure 2). Similar xyloside-mediated decreases in macrophage-associated apoE (*29*) and in a heparin-releasable fraction of apoE from the HepG2 cell surface (*12*) have been reported. Maintenance of β-D-xyloside in the growth medium also increased apoE secretion by 4.3-fold in HepG2 cells (Figure 2), suggesting that cell surface GAGs represent a substantial reservoir for secreted apoE. This is in agreement with the findings of Lucas and Mazzone (*29*) with J774 macrophage transfected with human apoE, but not with the observations of Lilly-Stauderman et al. (*12*) who reported no significant change in the secretion of apoE from HepG2 cells in response to xyloside. Differences in experimental procedures may explain the discrepancy with the latter study (*12*) which was the only one to maintain cells in serum-free medium for 24 h prior to the experiment. The sensitivity of apoE secretion to interleukin-1, interferon-γ and basic fibroblast growth factor (*30*), tumor necrosis factor (*31*) and insulin (*32*), 3,3′-5-triiodothyronine (*33*), and growth hormone (*34*) in various cell types could readily explain the discrepant results seen in the absence of serum.

In HepG2 cells the amounts of apoE at the cell surface probably represents a small proportion of the total secreted pool of apoE. In support of this Ji et al. (*5*) found increases of only 12% in the medium apoE following 2 h incubations of HepG2 cells with heparinase or suramin. This suggests that in our studies with xyloside the increase in secreted apoE far exceeds the amount lost from the cell surface pool. This could be explained by the xyloside-induced loss of a rapidly recycling apoE−GAG pathway. The pathway may depend on the presence of LRP or other receptors and would ultimately result in the degradation of apoE, thereby preventing its build-up in the extracellular medium. Alternatively, the secretion of GAGs and apoE may be intimately linked intracellularly. In support of this, xylosides do increase the overall synthesis and secretion of GAGs to the extracellular

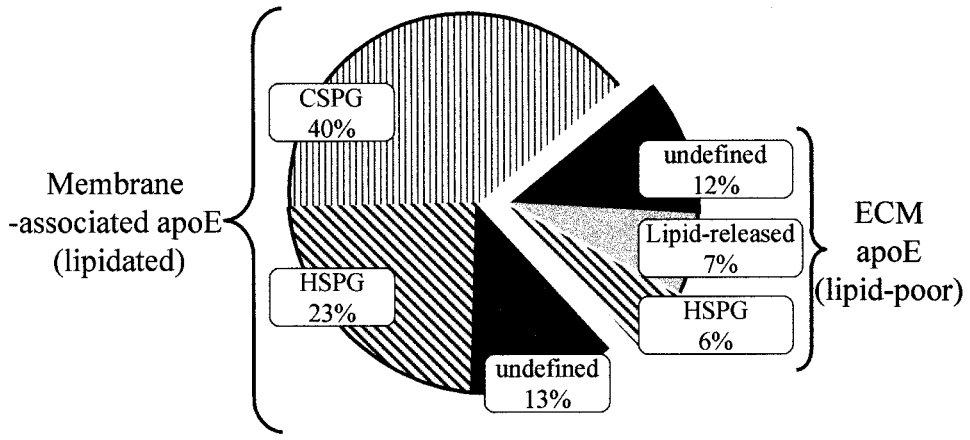

FIGURE 6: Distribution of apoE on the HepG2 cell surface. ApoE of the HepG2 ECM is lipid poor and can be divided into HSPG-bound (6% of the total cell surface pool) and lipid-releasable (7.0%) pools as well as a population that associates with the matrix in an unidentified manner (*14*). Plasma membrane-associated apoE contains lipids and can be divided into CSPG- (40%) and HSPG-associated (23%) populations. ApoE that binds to the plasma membrane in an unidentified manner accounts for the remaining cell surface apoE.

medium (*22*) which in turn would explain the increased secretion of apoE.

The types of GAGs found in liver are, in order of decreasing concentration, heparan sulfate (more than 60% of total GAGs), dermatan sulfate, and chondroitin sulfate isomers (*23*). Numerous studies with heparinase have implicated cell surface HSPG in the binding and uptake of many ligands including apoE (*12*−*14*) and apoE-enriched remnant particles (*4*, *5*, *35*). In our studies 3 or 10 units/mL of heparinase decreased cell surface apoE by only 23% in HepG2 cells (Figure 3). In agreement, heparin treatment decreased cell surface apoE by 25−30% (Table 1). This suggests that the majority of apoE may bind to alternate sites on the cell surface, possibly other GAGs. CSPGs are synthesized and secreted by HepG2 cells (*36*−*38*) and rat hepatocytes (*39*, *40*), and exogenous chondroitin sulfates have been shown to release apoE from HepG2 cells (*12*). Our data (Figure 3 and Table 1) collectively indicate that the majority of cell surface apoE associates with species of CSPG that contain galactosamine-4-sulfate. The specific positioning of sulfate residues on the polysaccharide chain of GAGs is important for the recognition of some ligands. Basic FGF, for example, requires 2-*O*-sulfation for recognition of HSPG (*41*, *42*), while CSPG fractions with elevated chondroitin 6-sulfate/chondroitin 4-sulfate ratios exhibit an increased affinity for native LDL (*43*).

The extent of GAG sulfation is an important factor in the interaction of ligands, including fibroblast growth factor (*44*) and hepatic lipase (*45*) with HSPG. The extent of the sulfation of CSPG/DSPG also appears to be important in the binding of LDL to fibroblasts and THP-1 cells (*46*). In contrast, the binding of apoE enriched β-VLDL to the surface of McA-RH7777 cells is relatively insensitive to chlorate requiring concentrations greater than 60 mM before a significant effect is observed (*5*). In our studies inclusion of 10 or 30 mM chlorate decreased cell surface apoE by about 25% at both concentrations while reducing ^{35}S-labeling by 37 and 67%, respectively (Figure 3). This indicates that a chlorate-sensitive pool, representing about 25% of the total cell surface apoE, and a substantially larger chlorate-resistant pool are present on the HepG2 cell surface. This is further supported by the degrees of sulfation of GAG species. HSPGs, although maintaining relatively high levels of sulfation (0.4−2 sulfates/disaccharide unit; *24*), account for only 25% of the total cell surface apoE. In contrast the poorly sulfated CSPGs (0.1−1.3 sulfates/disaccharide unit; *24*) account for approximately 50% of cell surface apoE. Therefore specific sites of sulfation, especially carbon 4 of galactosamine, may be more important than the extent of GAG sulfation for the maintenance of cell surface apoE.

The association of apoE with HSPG involves an electrostatic interaction between arginine and lysine residues in the receptor binding region (residues 142−147) and C-terminus (residues 202−243) of apoE and the negatively charged carboxyl and sulfate groups of HSPG (*25*, *26*). The effects of monoclonal antibodies, which recognize specific domains of apoE, on the interaction between apoE and heparin have been examined previously (*26*). In these studies the binding of lipid-free apoE to HSPG and CSPG was efficiently inhibited (60−70%) by antibodies which recognize the receptor-binding domain of apoE and only partially (∼40%) by 3H1 (which recognizes an epitope within residues 243−272 of the C-terminal tail; Figure 4). In our studies mAb against the receptor binding region, 1D7 and 2E8, decreased the binding of apoE to HSPG and CSPG by 65−70%. In contrast 3H1 more efficiently inhibited the binding of lipid-free apoE to CSPG and DSPG (77 and 73%, respectively) than to HSPG (54%). Association of apoE with DMPC vesicles severely decreased binding to the immobilized glycosaminoglycans by almost 90% (Figure 4) when compared to lipid-free controls. We have previously reported that apoE of the ECM is lipid-poor and that increasing the lipid complement results in decreased binding to the ECM (*14*). The C-terminal tail may therefore be important for the interaction of lipid-poor apoE with GAGs of the ECM.

The distribution of apoE on the HepG2 cell surface as determined by our immunometric analysis is summarized in Figure 6. ApoE of the ECM accounts for about 24% of the cell surface pool (*14*) and includes HSPG-associated and lipid-releasable populations as well as a pool that associates with the ECM in an unidentified manner. These populations represent approximately 6, 7, and 12%, respectively, when expressed as a proportion of the cell surface pool. The remainder of cell surface apoE is distributed on plasma membrane GAGs (40% CSPG, 24% HSPG). Approximately 12% of the plasma membrane pool apoE is not accounted

for in our enzymatic and matrix analysis. This population may include apoE bound to cell surface receptors or laminin (*47*). While Figure 6 represents apoE that can be detected by incubation with antibody, it is also possible that a sequestered pool of apoE exists at the cell surface that is not accounted for in Figure 6. ApoE of the ECM is lipid poor as judged by its poor reactivity with 3H1, a monoclonal antibody against an epitope contained within the C-terminal lipid-binding domain of apoE. In matrix-depleted cells the 3H1 immunoreactivity of plasma membrane-associated apoE is unaffected by the addition of lipids, indicating that it represents a lipid-rich fraction of apoE.

In conclusion we have found that apoE of the HepG2 cell surface associates mainly with CSPG and that apoE present at the plasma membrane is lipidated. Current work is directed at the identification of the core protein containing these GAGs. In preliminary experiments we have found that cell surface apoE can be donated to newly secreted lipoprotein particles, a property not previously recognized, but in agreement with the recent report that VLDL secretion is greatly reduced in hepatocytes derived from apoE-deficient mice (*48*).

ACKNOWLEDGMENT

We thank Drs. R. McPherson, R. Milne, and Z. Yao for useful discussions and comments on the manuscript.

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BI9819778