Endogenous apoE expression modulates HDL_3 binding to macrophages

Chen-Yi Lin, Madhuri Lucas, and Theodore Mazzone[1]

Departments of Medicine and Biochemistry, Rush Medical College, Chicago, IL 60612

Abstract We have previously shown that expression of a human apoE cDNA in J774 macrophages enhances cholesterol efflux to HDL_3. We have also shown that endogenous apoE expression produces a cell surface pool of apoE associated with proteoglycans. In this series of experiments, we first demonstrate the presence of a cell surface proteoglycan-associated apoE pool in human monocyte-derived macrophages. We then examine the hypothesis that endogenous expression of apoE modulates HDL_3 binding to macrophages, thereby, accounting for enhanced cholesterol efflux to HDL_3, specifically examining a role for the cell surface pool. Enhanced binding of apoE-free human HDL_3 to apoE-expressing macrophages, compared to non-expressing macrophages, was observed at 37°C and 4°C. The enhanced binding was not due to apoE secreted into the medium, as determined by experiments utilizing conditioned medium from apoE-secreting cells. Removal of the cell surface pool of apoE, however, substantially reduced the incremental HDL_3 binding produced by apoE expression. Cellular cholesterol mass measurements demonstrated that experimental conditions that reduced HDL_3 binding to apoE-expressing macrophages, did not substantially reduce cholesterol efflux to HDL_3. In summary, our results document a clear role for cell surface pool of apoE in modulating HDL_3 interaction with macrophages. The enhanced binding, however, does not appear to be a major mechanism contributing to the increased cholesterol efflux to HDL_3, which results from endogenous macrophage expression of apoE.—**Lin, C-Y., M. Lucas, and T. Mazzone.** Endogenous apoE expression modulates HDL_3 binding to macrophages. *J. Lipid Res.* 1998. **39:** 293–301.

Supplementary key words macrophages • apolipoprotein E • HDL_3 • cell cholesterol homeostasis

Macrophage-specific expression of apoE in vivo has been shown to protect against the development of atherosclerotic lesions, even in the presence of high levels of circulating atherogenic lipoproteins (1, 2). Many potential mechanisms can be considered for this protective effect, including apoE interaction with platelets (3), lymphocytes (4, 5), arterial smooth muscle cells or extracellular matrix (6, 7). One mechanism of particular interest, given the prominence of macrophage-derived foam cells in the developing atherosclerotic plaque lesion, is the increase in cholesterol efflux from macrophages which has been observed as a function of apoE expression. This has been clearly demonstrated utilizing the J774 macrophage cell model, which does not express its endogenous apoE gene. Expression of a human apoE cDNA in these cells has been shown to enhance cholesterol efflux to HDL_3 compared to parent macrophages which were identical in every way; except for apoE expression (8). ApoE produced by primary cultures of human monocyte-derived macrophages (9) and the RAW 64 macrophage cell line (10) has also been associated with reduction in macrophage cholesterol content and enhanced cholesterol efflux.

We have previously suggested a number of potential mechanisms for the enhancement of cholesterol efflux to HDL_3, which is observed as a function of apoE expression in macrophage cells (8). These included alteration of second messenger pathway signaling, alteration of subcellular cholesterol transport or plasma membrane composition, and alteration of HDL_3/macrophage interaction. In this series of experiments, we have evaluated the latter mechanism, specifically examining HDL_3 binding as a function of endogenous apoE expression in macrophages. This line of investigation was of particular interest, given our recent observation that endogenous apoE expression produced the appearance of a proteoglycan-associated pool of apoE on the macrophage cell surface.

Abbreviations: βDX, 4-methylumbelliferyl 1-β-d-xyloside; CE, cholesteryl ester; FC, free cholesterol; ALDL, acetylated low density lipoprotein; HDL, high density lipoprotein; BSA, bovine serum albumin; FBS, fetal bovine serum.

[1]To whom correspondence should be addressed.

EXPERIMENTAL PROCEDURES

Materials

[^{125}I]sodium iodine (17 Ci/mg) was purchased from DuPont NEN (Wilmington, DE). Iodo beads were from Pierce. PD-10 gel filtration columns were obtained from Pharmacia (Piscataway, NJ). Heparin-Sephadex column matrix was obtained from Bio-Rad. Heparinase 1 (EC 4.2.2.7), βDX, BSA, aprotinin, leupeptin, and benzamidine were purchased from Sigma (St. Louis, MO). Goat-derived anti-human apoE antiserum was purchased from International Immunology Co. (Murrieta, CA). All organic solvents, including isopropanol, methanol, hexane, and chloroform were from Fisher (Pittsburgh, PA). Dulbecco's modified Eagle's medium, FBS, and neomycin (G418) used for tissue culture were obtained from Gibco (Grand Island, NY).

Isolation and iodination of apoE-free HDL

Human plasma was obtained from healthy normolipemic donors. HDL_3 was isolated from plasma by sequential isopycnic ultracentrifugation at d 1.125–1.250 g/ml (8). The purity of the isolated HDL_3 was assessed by agarose gel electrophoresis and lipid staining. After extensive dialysis in 1 mm sodium phosphate buffer, HDL_3 was subjected to heparin affinity chromatography to remove any residual apoE. The apoE-deficient HDL_3 was dialyzed again in 100 mm sodium phosphate buffer, and concentrated using an Amicon concentrator. ApoE-free HDL_3 was iodinated according to Markwell (11). Briefly, Iodo beads were first rinsed with 100 mm sodium phosphate buffer and mixed with [^{125}I]NaI at room temperature for 10 min. ApoE-free HDL_3 was added to the mixture and incubated in the absence of any reducing reagents for 40 min. After this incubation, free [^{125}I]NaI was removed from the radiolabeled HDL_3 by gel filtration chromatography through a PD-10 column, followed by extensive dialysis at 4°C in PBS in the presence of 1 mm EDTA. After dialysis, 98% of [^{125}I]radiolabel in the HDL_3 preparation was precipitated by 20% TCA at 4°C, and 1% of the [^{125}I]radiolabel in the TCA-precipitated fraction was extracted into the lipid phase by the method of Bligh and Dyer (12).

HDL_3 binding

The J774 cells stably transfected to express a wild type human apoE cDNA, (apoE expressing) and the control parental line transfected with a neomycin resistance construct only (non-expressing) have previously been characterized in detail (8, 13–15). Both apoE expressing and non-expressing cells were selected and maintained in DMEM containing 10% FBS in the presence of 0.5 mg/ml G418. G418 was removed from the growth medium 1 week before the initiation of experiments.

J774 cells were plated in 24-well plates at 0.2 million cells/well in DMEM + 10% FBS for 48 h. On the day of experiment, the confluent cells were washed twice with PBS containing 0.1% BSA at room temperature, followed by incubation in DMEM + 0.2% BSA at 37°C for 60 min. After this incubation, the cells were washed twice in DMEM + 0.1% BSA and chilled on ice for 30 min. The washing medium was aspirated and [^{125}I]-labeled apoE-free HDL_3 was then added to the cells in the presence or absence of 20-fold excess of unlabeled apoE-free HDL_3. The pH of the binding medium (DMEM + 0.1% BSA) was adjusted to 7.4 using HEPES, prior to adding to the cells. The binding incubation was carried out either at 4°C for 120 min or at 37°C for 60 min, and terminated by aspirating the ^{125}I-labeled HDL_3-containing medium and washing the cells three times, with ice-cold PBS + 0.2% BSA followed by three additional washes with cold PBS alone. The cells were then dissolved in 0.1 N NaOH, and aliquots of sample were quantitated for protein content using method of Lowry et al. (16) and [^{125}I] radioactivity. Throughout the manuscript, results of representative experiments are shown.

Measurement of proteoglycan-associated apoE in human monocyte-derived macrophages

Human monocytes were obtained by elutriation. Cells were >90% monocytic (as determined by differential counts of Wright-stained smears) and incubated as described previously (17). Briefly, monocyte-derived macrophages, 2.0 million cells/well, were grown in DMEM containing 20% FBS and 10% human AB serum for 7 days. For the last 3 days of the 7-day incubation, 1 mm βDX was added to half of the cultures. On day 7, cells were pulse-labeled for 60 min. Pulse medium contained 100 μCi/ml [^{35}S]methionine with 1 μm unlabeled methionine in methionine-free DMEM. Selected cultures were treated with heparinase (3 U/ml) during the 60-min pulse period. Chase medium contained 500 μm unlabeled methionine. At the end of the chase period (45 min) apoE secreted into the medium was quantitatively immunoprecipitated and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis as described in detail (13–15). The apoE bands were localized using a radiofluorescent image scanner, and quantitated using ImageQuant software. All immunoprecipitations were conducted using equal numbers of total trichloroacetic acid-precipitable counts; the quantitation of secreted apoE is, therefore, already corrected for total labeled secreted protein. In multiple experiments performed separately in cells labeled with [^{35}S]sodium sulfate, we have detected a 30–80% de-

crease in trichloroacetic acid-precipitable cell associated radioactivity in response to a 72-h incubation with βDX or a 60-min exposure to 3 U/ml of heparinase.

Other methods

Cellular free and total cholesterol were measured using gas–liquid chromatography as described previously (8). ALDL was prepared by the repeated addition of acetic anhydride to low density lipoprotein (d 1.019–1.063 g/ml) as described (8). Statistical comparisons were performed using a two-tailed *t*-test.

RESULTS

Identification of a proteoglycan-associated cell surface pool of apoE in human monocyte-derived macrophages

We have previously characterized a pool of apoE associated with cell surface proteoglycans in J774 macrophages that were transfected to constitutively express a human apoE cDNA (14). Recently it was reported that such a pool could not be identified in macrophages isolated from the mouse peritoneal cavity (18). To investigate the physiological relevance of our observations in the J774 cell model, we examined this same issue in primary cultures of human monocyte-derived macrophages, using identical experimental protocols as those utilized for the demonstration of a cell surface proteoglycan-associated pool in apoE expressing J774 cells. Treatment with exogenous heparinase was used to hydrolyze cell surface heparan sulfate. βDX substitutes for the core protein of proteoglycans and significantly reduces their synthesis and appearance at the cell surface (14). As shown in **Fig. 1**, treatment with heparinase or βDX increased the release of apoE from human monocyte-derived macrophages by 2.1- and 4.4-fold, respectively. The lack of additional effect of extracellular heparinase treatment, in cells preincubated with βDX, suggests that this latter agent completely inhibited cell surface expression of the substrate for this enzyme. Our results indicate that a significant amount of apoE synthesized by human monocyte-derived macrophages is sequestered by proteoglycans on the cell surface. These results are entirely consistent with our previously reported results using the transfected J774 model.

Binding of apoE-free HDL_3 to apoE expressing and non-expressing macrophages

We took advantage of the J774 model to investigate the effect of apoE expression on the binding of HDL_3 to macrophages. This model afforded the opportunity to compare HDL_3 binding to macrophages that differed only in the expression of apoE. The binding of ^{125}I-labeled HDL_3 to J774 macrophages was examined at 4°C in the presence and absence of a 20-fold excess of unlabeled HDL_3 in apoE expressing and non-expressing cells. As shown in **Fig. 2**, labeled HDL_3 binds both apoE expressing and non-expressing macrophages, in a dose-dependent and saturable fashion. ApoE expressing cells (panel A), however, bind more HDL_3 in a specific manner, compared to non-expressing cells (panel B). Binding curves in the presence of a 20-fold excess of unlabeled HDL_3 (non-specific binding) were similar in apoE expressing and non-expressing macrophages. The data from the specific binding curves were subjected to Scatchard analysis and this result is shown in Fig. 2C. Both apoE expressing and non-expressing cells gave rise to curvilinear plots most consistent with binding at two sites with different affinity. As can be seen, the major difference between apoE expressing and non-expressing cells appears to be an increased binding capacity in the lower affinity binding site. Binding experiments conducted in cells that were cholesterol-loaded by pre-incubation in ALDL showed

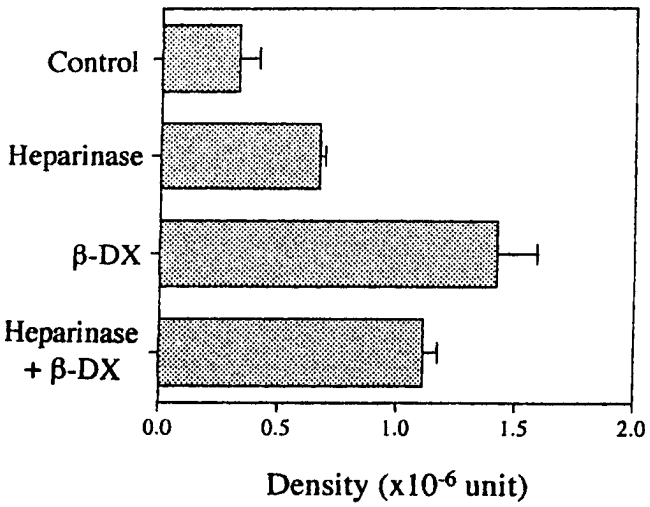

Fig. 1. Effect of proteoglycan depletion on the release of newly synthesized apoE from human monocyte-derived macrophages. Human monocyte-derived macrophages were plated and grown as described in Experimental Procedures. βDX (1 mm) was added, where indicated, for 72 h prior to the labeling of cells. On the day of the experiment, the cells were washed and pulse-labeled for 60 min with 100 μCi/ml of [^{35}S]methionine and with 1 μm cold methionine at 37°C. Where indicated, heparinase (3 units/ml) was also included. After the labeling period, the cells were chased in the presence of 500 μm of unlabeled methionine for 45 min. Equal amounts of trichloroacetic acid-precipitable radioactivity were immuno-precipitated using a goat-derived, human apoE antiserum. The apoE bands were detected using a radio-fluorescent scanner and quantitated using ImageQuant software. The results shown are the mean ± SD of triplicate samples.

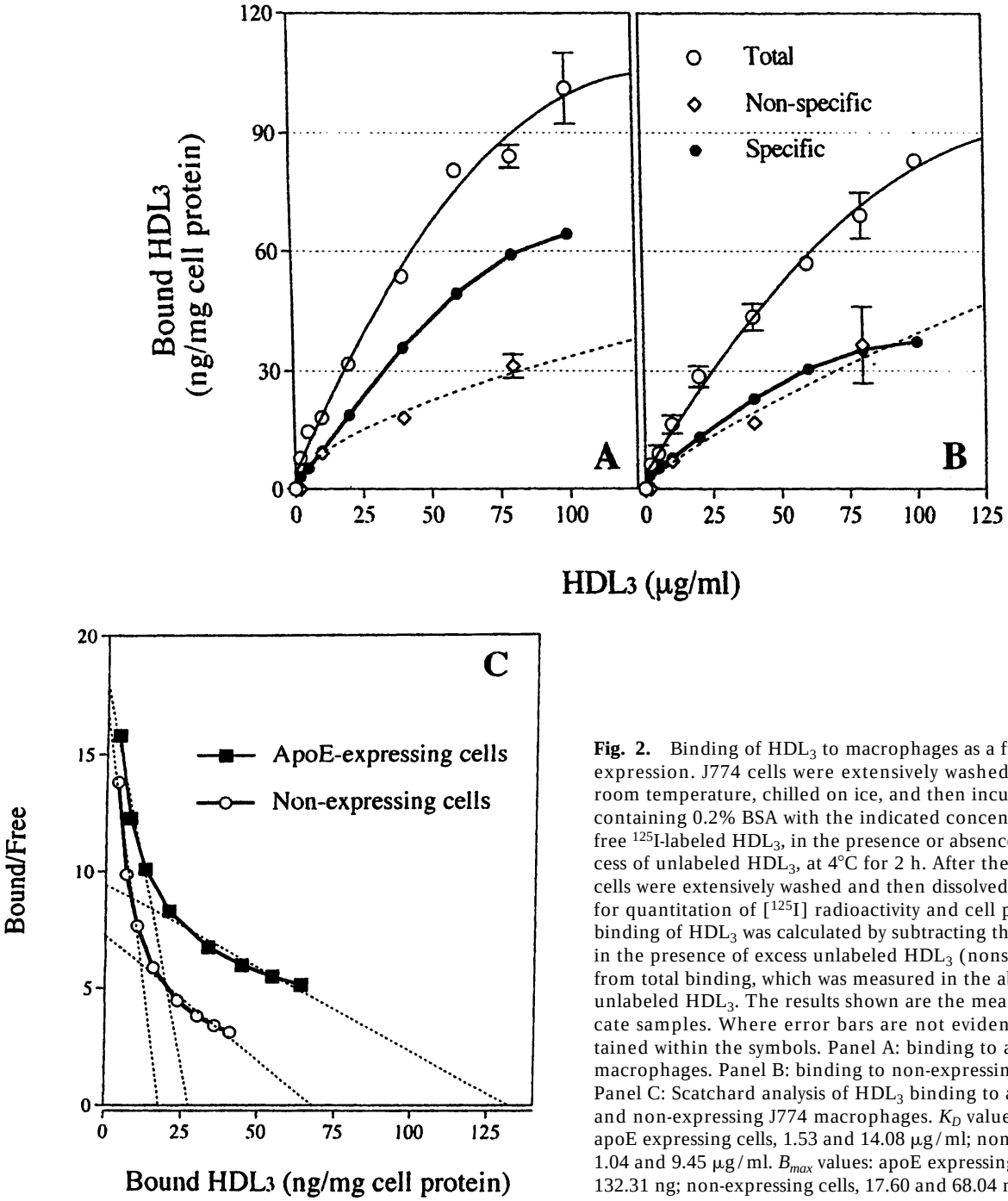

Fig. 2. Binding of HDL_3 to macrophages as a function of apoE expression. J774 cells were extensively washed with DMEM at room temperature, chilled on ice, and then incubated in DMEM containing 0.2% BSA with the indicated concentration of apoE-free ^{125}I-labeled HDL_3, in the presence or absence of a 20-fold excess of unlabeled HDL_3, at 4°C for 2 h. After the incubation, the cells were extensively washed and then dissolved in 0.1 N NaOH for quantitation of [^{125}I] radioactivity and cell protein. Specific binding of HDL_3 was calculated by subtracting the HDL_3 binding in the presence of excess unlabeled HDL_3 (nonspecific binding) from total binding, which was measured in the absence of excess unlabeled HDL_3. The results shown are the mean ± SD of triplicate samples. Where error bars are not evident, they are contained within the symbols. Panel A: binding to apoE expressing macrophages. Panel B: binding to non-expressing macrophages. Panel C: Scatchard analysis of HDL_3 binding to apoE expressing and non-expressing J774 macrophages. K_D values are as follows: apoE expressing cells, 1.53 and 14.08 μg/ml; non-expressing cells 1.04 and 9.45 μg/ml. B_{max} values: apoE expressing cells 27.59 and 132.31 ng; non-expressing cells, 17.60 and 68.04 ng.

similar differences in HDL_3 binding as a function of apoE expression (not shown).

Generally, similar binding characteristics were observed when the binding incubations were performed at 37°C for 1 h (**Fig. 3**). Compared with the experiments conducted at 4°C, an increase in temperature increased the binding of HDL_3 to both cell types, reflecting either more HDL_3 binding sites expressed on the cell surface at that temperature, internalization of HDL_3, or the effect of continuing apoE secretion (at 37°C) on HDL_3 particle composition. Like the experiments performed at 4°C, however, apoE expressing cells bind more HDL_3 than non-expressing cells.

Effect of secreted apoE on the interaction of HDL_3 with macrophages

ApoE enhancement of the binding of HDL_3 to the macrophage surface could result from apoE seques-

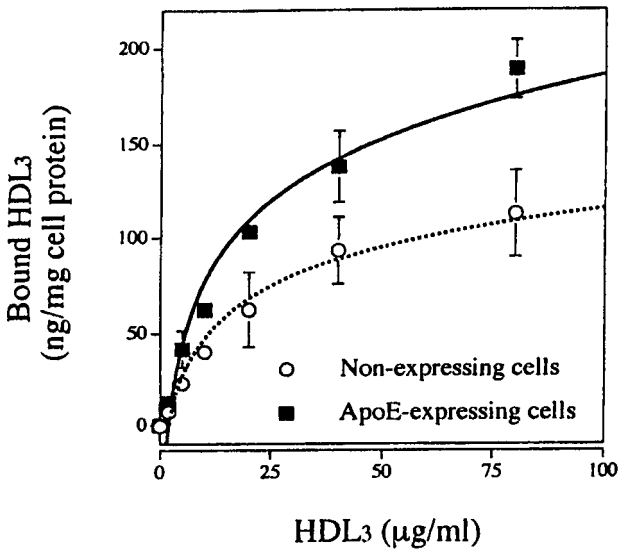

Fig. 3. Binding of HDL_3 to macrophages at 37°C. HDL_3 binding experiments were performed as described in Fig. 2, except that the cells were incubated with labeled HDL_3 at 37°C for 1 h. The results shown are the specific HDL_3 binding and represent the mean ± SD of triplicate samples. Where error bars are not evident, they are contained within the symbols.

tered on the macrophage cell surface or apoE secreted into the medium. Modification of HDL_3 composition by secreted apoE could also contribute to the enhanced binding of HDL_3 to apoE expressing cells observed at 37°C compared to 4°C (see above). An experiment was conducted to specifically study the effect of apoE, secreted by apoE expressing cells, on the interaction of labeled HDL_3 with non-expressing macrophages. Conditioned media were collected from apoE expressing cells or non-expressing cells after a 1-; 2-; or 3-h incubation, and then added to non-expressing cells with ^{125}I-labeled HDL_3, in the presence or absence of a 20-fold excess of unlabeled HDL_3. Specific binding in the presence of each of these conditioned media was compared to the binding observed when HDL_3 was added in fresh medium (not previously exposed to macrophages). The results of this experiment are shown in **Table 1**, in which binding in the presence of each type of conditioned medium is expressed as a percentage of the binding observed in fresh medium. As shown, comparable HDL_3 binding was observed to non-expressing cells exposed to conditioned media from either apoE expressing or non-expressing cells; also, these values also tended to be somewhat reduced compared to binding observed in fresh medium. These data indicate that apoE secreted into the medium does not contribute to the enhanced HDL_3 binding observed in apoE expressing cells; and, therefore, implicate the cell surface pool of apoE as important.

TABLE 1. Effect of secreted apoE in J774 cell conditioned medium on the interaction of HDL_3 with non-expressing macrophages

Conditioned Medium from	Incubation Time		
	1 h	2 h	3 h
	% change		
Expressing cells	89.7 ± 1.0	92.8 ± 4.0	83.5 ± 0.9
Non-expressing cells	84.3 ± 4.3	90.8 ± 2.1	76.8 ± 3.4

Conditioned media (DMEM + 0.1% BSA) were collected from either apoE expressing or non-expressing cells after a 1-, 2-, or 3-h incubation and then added to non-expressing cells with labeled HDL_3 (50 μg/ml plus or minus a 20-fold excess of unlabeled HDL_3). Fresh medium, without exposure to J774 cells, was utilized in the binding studies as a control and the specific HDL_3 binding in the presence of each type of conditioned medium is expressed as a percentage of change compared to fresh medium. Values shown are the mean ± SD from triplicate samples.

Effect of the cell surface apoE pool on HDL_3 interaction with macrophages

In order to more directly investigate a potential role of cell surface apoE for the enhanced HDL_3 binding to apoE expressing cells, cell surface apoE was reduced by depleting cell surface proteoglycans. Cell surface proteoglycans were depleted by incubation with heparinase. In experiments conducted with cells labeled to equilibrium with [^{35}S]sodium sulfate, we measured a 68.2 ± 4.7% increase in the release of radioactivity into the medium after the heparinase treatment protocol described in **Table 2**. Non-expressing cells were also studied so we could be certain that any changes produced in HDL_3 binding by heparinase treatment reflected removal of apoE and not removal of proteoglycans. As shown in Table 2, in apoE expressing cells, pretreatment with heparinase significantly decreased the binding of ^{125}I-labeled HDL_3. In contrast, heparinase treatment did not influence the binding of HDL_3 to non-expressing cells. Heparinase treatment reduced

TABLE 2. Effect of heparinase treatment on the interaction of HDL_3 with apoE expressing and non-expressing macrophages

Treatment	HDL_3 Bound	
	ApoE Expressing	Non-Expressing
	ng/mg cell protein	
Control	39.50 ± 6.22[ab]	23.00 ± 2.34[a]
Heparinase	28.78 ± 1.68[b]	24.61 ± 2.07

J774 macrophages were washed with DMEM + 0.2% BSA and incubated with heparinase (12 units/ml) at 37°C for 2 h as indicated. After this incubation, the cells were extensively washed and labeled HDL_3 (50 μg/ml ± a 20-fold excess of unlabeled HDL_3) was added for measurement of specific HDL_3 binding as previously described. The data shown are the mean ± SD from triplicate samples.

[a] $P < 0.05$, apoE expressing versus non-expressing cells.

[b] $P < 0.05$, control versus heparinase treatment.

the increment in binding of HDL_3 attributable to apoE expression by 75%. These results implicate the cell surface pool of apoE as primarily mediating the increased binding of HDL_3 to apoE expressing cells.

We have previously shown that incubation in lactoferrin can also increase medium apoE and reduce cellular apoE in macrophage cultures, primarily by displacing apoE from its cell surface pool (14). We, therefore, examined the effect of lactoferrin on HDL_3 binding in apoE expressing and non-expressing cells as an alternate means of assessing the role of a cell surface pool of apoE in modulating HDL_3 binding. As shown in **Table 3**, lactoferrin had no effect on HDL_3 binding to non-expressing cells, but significantly reduced binding to apoE expressing cells. This result is completely consistent with the data and conclusions from Table 2.

Effect of reducing HDL_3 binding on cholesterol efflux to HDL_3

We have previously shown that apoE expression enhances cholesterol efflux from J774 macrophages, particularly in the presence of an exogenous acceptor, e.g., HDL_3 (8). One potential mechanism for this enhanced HDL_3-mediated efflux is the enhanced binding of HDL_3 to the macrophage surface (demonstrated in the above experiments), which occurs in the presence of the cell surface apoE pool. We, therefore, conducted an experiment to examine the relationship between increased HDL_3 binding and increased cholesterol efflux in apoE expressing cells. Both apoE expressing and non-expressing cells were preincubated with ALDL, in order to increase cellular free cholesterol and cholesteryl ester content before the start of the experiment. We depleted the cell surface pool of apoE by a 72-h preincubation in βDX and by heparinase treatment; non-expressing cells were also treated with heparinase and βDX. Cells were then incubated for 24 h, in the presence or absence of HDL_3 at 400 μg/ml before being harvested. The results of this experiment are shown in **Table 4**. Consistent with our previous observations (8), as well as the observations of others (19), the addition of HDL_3 had little effect on cellular free cholesterol or cholesteryl ester content in non-expressing cells, in either control (proteoglycan-replete) or proteoglycan-depleted cells. In examining apoE expressing cells, and comparing changes in cellular cholesterol content after the 24-h incubation in the presence or absence of HDL_3, an approximately similar decrement is produced by HDL_3 in control cells (proteoglycan-replete) and in cells depleted of proteoglycans. Control cells contained 51.1 μg/mg of total cholesterol after 24 h without HDL_3, and 39.7 μg/mg after 24 h in HDL_3, a 22% fall (11.4 μg/mg). In proteoglycan-depleted cells, 69.4 μg/mg of total cholesterol is measured after 24 h in the absence of HDL_3 and 56.8 μg/mg in the presence of HDL_3, an 18% fall (12.6 μg/mg). These data do not support a major role for enhanced binding of HDL_3 to macrophages, mediated by the cell surface pool of apoE, in the enhanced cholesterol efflux from macrophages to HDL_3 which occurs as a function of apoE expression.

TABLE 3. Effect of lactoferrin treatment on the interaction of HDL_3 with expressing and non-expressing macrophages

	HDL_3 Bound	
Treatment	ApoE Expressing	Non-expressing
	ng/mg cell protein	
Control	63.59 ± 1.63^{ab}	26.57 ± 1.70^{a}
Lactoferrin	47.73 ± 1.21^{b}	25.56 ± 1.13

J774 macrophages were washed with DMEM + 0.2% BSA and incubated with lactoferrin (250 μg/ml) for 60 min at 37°C. After this incubation, the cells were extensively washed and labeled HDL_3 (50 μg/ml ± a 20-fold excess of unlabeled HDL_3) was added for measurement of specific HDL_3 binding as previously described. The data shown are the mean ± SD of triplicate samples.

[a] $P < 0.005$, apoE expressing versus non-expressing cells.

[b] $P < 0.005$, control versus lactoferrin treatment.

DISCUSSION

There is now convincing evidence in vivo that establishes that macrophage expression of apoE in the vessel wall is anti-atherogenic (1, 2). There are several lines of in vitro investigations that support this conclusion, as well as provide possible mechanisms for the anti-atherogenic properties of macrophage-derived apoE (20). ApoE in the vessel wall could influence the aggregability of platelets (3) or the proliferation of lymphocytes (4, 5). ApoE interaction with extracellular matrix could influence the retention of lipoproteins by matrix or the availability of cytokines and growth factors, which also have affinity for matrix components (6). Further, macrophage interaction with matrix components could modulate the regulatory effects of matrix on arterial smooth muscle cell growth and proliferation (7). However, given the fact that foam cells (i.e., arterial smooth muscle cells and macrophages engorged with cholesterol) are a hallmark of the plaque lesion, in vitro studies showing that apoE influences cellular cholesterol balance are of particular interest. ApoE has been shown to enhance cholesterol efflux from both arterial smooth muscle cells and macrophages (21, 22). In macrophages, endogenous expression of apoE has been linked to enhanced cholesterol efflux (8–10). Observations using J774 cells transfected to express physiologic amounts of human apoE have demonstrated a cause

TABLE 4. Effect of decreasing HDL_3 binding on HDL_3-mediated efflux from macrophages

Cells	ApoE Expressing Cells		Non-Expressing Cells	
	24 h − HDL_3	24 h + HDL_3	24 h − HDL_3	24 h + HDL_3
	μg lipid/mg cell protein			
Control				
FC	39.5 ± 1.9	33.8 ± 2.9	47.4 ± 1.2	48.3 ± 2.7
CE	11.6 ± 2.8	5.9 ± 1.3	12.9 ± 1.0	15.3 ± 1.6
Proteoglycan-depleted				
FC	45.1 ± 1.5	42.7 ± 2.5	48.1 ± 1.0	47.8 ± 3.1
CE	24.3 ± 3.2	14.1 ± 1.7	14.7 ± 2.9	18.8 ± 2.2

ApoE expressing and non-expressing macrophages were preincubated with ALDL (80 μg/ml) and incubated in the presence or absence of βDX (1 mm) for 72 h as indicated. Cells treated with βDX were also treated with heparinase (3 units/ml at 37°C for 1 h). All cells were then extensively washed and incubated for an additional 24 h in the presence or absence of HDL_3 (400 μg/ml) and ± βDX (1 mm) before being harvested for measurement of free cholesterol, total cholesterol, and cell protein. The data shown are expressed as μg of lipid per mg cell protein and represent the mean ± SD of triplicate samples.

and effect relationship between endogenous apoE expression and cholesterol efflux (8). Therefore, an important mechanism to be considered for the antiatherogenic effect of apoE is its ability to enhance cholesterol efflux from the cells of the vessel wall.

The mechanism by which expression of apoE enhances cholesterol efflux from macrophages requires additional investigation. Because HDL_3-mediated efflux likely involves initial anchoring of HDL_3 to the macrophage cell membrane, we examined the hypothesis that apoE expression enhances cholesterol efflux by enhancing HDL_3/macrophage interaction. This mechanism was of additional interest because of the recent demonstration that macrophages express a cell surface pool apoE (14). The data in Fig. 1 show that apoE is sequestered in a cell surface proteoglycan-associated pool in human monocyte-derived macrophages. This observation establishes a complete analogy between J774 macrophages transfected to express human apoE and human monocyte-derived macrophages for cell surface apoE expression. These results are different from those recently reported for mouse peritoneal macrophages; in which a cell surface pool of apoE could not be identified (18). This difference could be due to differences in experimental approach or differences in cell type, suggesting that mouse peritoneal macrophages may not be a good model for human macrophages in this regard. However, it remains unclear why mouse peritoneal macrophages do not express a cell surface pool of apoE, given the observation that most macrophage cell types (including primary cultures and cell lines) produce abundant cell surface heparan sulfate and chondroitin sulfate proteoglycans and macrophage-derived apoE could potentially associate with either of these proteoglycan species (23–29). The data in Fig. 2 document an increased binding of HDL_3 to macrophages as a function of apoE expression at 4°C, and Fig. 3 demonstrates increased binding of HDL_3 as a function of apoE expression at 37°C. The Scatchard analyses of the specific binding curves (shown in Fig. 2C) are curvilinear for both apoE expressing and non-expressing cells, suggesting the presence of two distinct binding sites with differing affinity for HDL_3. These analyses also indicate that the increased binding of HDL_3 that results from apoE expression is due to an increase in the maximal binding capacity of the lower affinity site. The higher binding at 37°C compared to 4°C could relate to the ongoing secretion of apoE at 37°C, with subsequent modification of HDL_3 composition leading to its enhanced binding at the cell surface. The data from Table 1, however, suggest that secreted apoE does not interact with HDL_3 in a way that increases its binding affinity for the macrophage. In support of this, increased binding at 37°C was also observed in non-expressing J774 cells. The most likely explanation, therefore, for increased cell-associated values at 37°C compared to 4°C, is the internalization of HDL_3 which can occur at the former temperature.

The data from Table 2 show that heparinase treatment decreases HDL_3 binding to apoE expressing cells, but has no effect on the binding of HDL_3 to non-expressing cells. This latter control is important, as it shows that hydrolysis of cell surface proteoglycans alone was not responsible for changing HDL_3 binding and, therefore, implicates the apoE associated with these proteoglycans. Treatment with heparinase reduced the incremental binding of HDL_3 due to apoE expression by approximately 75%, suggesting that HDL_3 interaction with apoE on the cell surface was the predominant mechanism for the enhanced binding of HDL_3 observed as a function of apoE expression. A similar conclusion can be made from the data presented in Table 3. There are several potential interac-

tions which can be considered between the apoE-containing particles on the macrophage cell surface, and the HDL_3 particle. ApoE on the cell surface could interact with surface lipids on the HDL_3 particle. Alternatively, because apoE is secreted from this macrophage cell model in association with lipid, the lipid present in the apoE–lipid particle on the cell surface could interact with apoA-I on the surface of HDL_3. Direct interaction between apoE and apoA-I is also possible, but all of these potential interactions require investigation.

Although apoE expression increases HDL_3 binding, this increased binding does not appear to be the major mechanism for the increased cholesterol efflux which occurs as a function of apoE expression, based on the data shown in Table 4. This conclusion is reinforced by the observation that HDL_3 also binds in a specific and saturable fashion to non-expressing J774 cells, but does not stimulate cholesterol efflux from this cell type. It can also be seen in Table 4 that apoE expressing proteoglycan-depleted cells have more cholesterol mass, both in the absence and presence of HDL_3, compared to apoE expressing control proteoglycan-replete cells. This effect of proteoglycan depletion was not observed in non-expressing cells and, therefore, likely relates to the loss of the cell surface pool of apoE. Potential mechanisms for this observation are multiple and are currently under investigation. However, in spite of this difference in absolute cholesterol mass, a similar percentage of total cellular cholesterol was lost as a function of the HDL_3 incubation; indicating that loss of the cell surface pool of apoE, and consequent reduction in HDL_3 binding, did not impair cholesterol efflux to HDL_3.

If differences in HDL_3 binding do not account for enhanced cholesterol efflux due to apoE expression, other mechanisms must be considered for the apoE-dependent efflux from macrophage-type cells. These mechanisms could include alteration of subcellular lipid transport, modification of plasma membrane composition to facilitate cholesterol desorption, or modulation of cell second messenger signaling pathways. Further, implications of the presence of an apoE pool on the surface of macrophages for other macrophage functions relevant to vessel wall biology need further consideration. The results of the current series of studies clearly show that cell surface apoE can influence macrophage interaction with HDL_3. The influence of this pool must also be considered for macrophage interaction with other lipoprotein particles, including modified forms of LDL, native LDL, or VLDL. In addition, the proteoglycan-associated pool of apoE on the macrophage cell surface could impact on the interaction of macrophages with the extracellular matrix. This could occur if cell surface apoE interacts directly with extracellular matrix components or blocks the interaction of macrophage cell surface proteoglycans with extracellular matrix components. The above issues are currently being investigated to further understand the role of macrophage-derived apoE in vascular wall homeostasis.

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