Expression of heterologous human apolipoprotein E by J774 macrophages enhances cholesterol efflux to HDL_3

Theodore Mazzone[1] **and Catherine Reardon**

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

Abstract Expression of apolipoprotein (apo) E by macrophages is tightly regulated by cellular cholesterol content. We have investigated a potential modulating role for apoE on macrophage cholesterol homeostasis by stably transfecting the J774 macrophage, which does not express its endogenous apoE gene, with a human apoE cDNA expression vector and comparing cholesterol homeostasis in this cell line with that of a control line transfected with the neomyocin resistance construct only. Incubation in serum-free medium after cholesterol loading produced no difference in cellular cholesterol content between apoE secreting and non-secreting J774 cells. Similarly, in serum-free medium there was no difference in the amount of radiolabeled cholesterol effluxed. Addition of cAMP or S58035 to cholesterol-loaded J774 cells did enhance efflux of radiolabeled cholesterol from apoE secreting compared to non-secreting macrophages but did not detectably alter cellular free cholesterol or cholesteryl ester mass. Incubation with HDL_3 alone, however, significantly decreased macrophage cholesteryl ester mass compared to a 24-h incubation in serum-free medium from 10.5 ± 3.9 to 3.2 ± 2.0 ($P < 0.01$) in apoE-secreting J774 cells. During a 24-h incubation in HDL_3, cholesteryl ester fell from 6.4 ± 2.4 to 0.8 ± 0.7 (Δ = 5.6 μg/mg) in apoE-secreting cells and from 9.3 ± 2.2 to 7.7 ± 1.9 μg/mg (Δ = 1.6 μg/mg) in non-secreting cells ($P < 0.005$ apoE-secreting vs. non-secreting cells). Drug-induced stimulation of cholesteryl ester hydrolysis (with cAMP) or inhibition of cholesterol esterification (with S58035) did not abolish the difference in cholesterol efflux to HDL_3 between apoE-secreting and non-secreting cells indicating that the effect of apoE on efflux is not due to alteration of free cholesterol–cholesteryl ester balance. These data indicate that secretion of endogenously synthesized apoE can enhance the loss of cellular cholesterol from cholesterol-enriched macrophages. The reciprocal regulation, represented by cholesterol modulation of apoE expression and apoE modulation of macrophage cholesterol balance, suggests a regulatory loop allowing macrophages to more efficiently function in order to maintain endogenous cellular and tissue cholesterol homeostasis.—**Mazzone, T., and C. Reardon.** Expression of heterologous human apolipoprotein E by J774 macrophages enhances cholesterol efflux to HDL_3. *J. Lipid Res.* 1994. **35:** 1345–1353.

Supplementary key words atherogenesis • high density lipoprotein

The macrophage is one of a number of extrahepatic cell types capable of synthesizing and secreting significant amounts of apoE (1–3) and the physiologic role(s) of apoE made by extrahepatic cells, including the macrophage, are still under investigation (4). A number of observations, however, suggest that macrophage apoE synthesis and secretion may play a role in modulating macrophage cholesterol balance. The expression of apoE by mouse peritoneal macrophages, human monocyte-derived macrophages, and the human monocyte-macrophage cell line THP1 is enhanced by increasing cellular cholesterol content (1, 5–7). This enhancement is achieved primarily at the level of apoE gene transcription and we have demonstrated an approximate 8-fold increase in this parameter after loading mouse peritoneal macrophages with cholesterol (6). In THP1 cells, which express both the apoE and the LDL receptor gene, apoE and LDL receptor gene expression vary inversely over identical ranges of cellular cholesterol content; i.e., the apoE gene responds to changes in cellular cholesterol content over an identical range as the LDL receptor gene; a paradigmatic sterol-responsive gene (7). These observations give rise to the hypothesis that apoE synthesis and secretion may function to facilitate the disposition of excess macrophage cholesterol. This proposed function for apoE could be of significance for maintaining the appropriate cholesterol balance of the artery wall.

Abbreviations: apo, apolipoprotein; HDL, high density lipoprotein; LDL, low density lipoprotein; ALDL, acetylated low density lipoprotein; DMEM, Dulbecco's modified Eagle's medium; BSA, bovine serum albumin; SDS, sodium dodecyl sulfate; ACAT, acyl-CoA:cholesterol acyltransferase.

[1]To whom correspondence should be addressed.

Cholesteryl ester-loaded macrophages are a hallmark of the atherosclerotic vessel wall plaque lesion (8). The source of this excess intracellular cholesterol is believed to be LDL, which has been oxidatively modified so that it is bound and internalized by the macrophage scavenger receptor (9). The cholesteryl ester stores of macrophages have been shown to undergo a continuous cycle of hydrolysis (to free cholesterol) and re-esterification (10). The hydrolytic step of this cycle is mediated by a neutral cytoplasmic cholesterol ester hydrolase, which is responsive to cAMP (11). The re-esterification step is mediated by ACAT (10). It has been shown that excess macrophage cholesteryl ester can be mobilized by extracellular cholesterol acceptors (e.g., human HDL_3), however, efflux agents do not directly stimulate the activity of cholesterol esterase or repress the activity of ACAT (10). Instead, in the presence of HDL_3, newly formed free cholesterol (from cholesteryl ester) is removed from the ACAT substrate pool, thereby suppressing re-esterification and promoting cholesteryl ester clearance. Effective mobilization of cholesteryl ester stores in macrophages, therefore, depends on the presence of an extracellular acceptor of free cholesterol, and the availability of a pool of cellular free cholesterol.

The proposal that apoE can influence macrophage cholesterol balance not only derives from the responsiveness of its expression to macrophage cholesterol content, but also from a number of other observations made in macrophages and other cell types. First, HDL_3-mediated efflux has been shown to be very active in cultures of primary macrophages (human monocyte-derived and mouse peritoneal) and in macrophage lines (THP1) that synthesize and secrete apoE (5–7). On the other hand, HDL_3-mediated efflux has been observed to be deficient in certain macrophage lines that do not express their endogenous apoE gene (e.g., J774 macrophages) (12). Second, Hara and Yokoyama (13) have shown that the addition of 10–30 μg/ml of delipidated apoE (isolated from VLDL) can promote cholesterol efflux from macrophages. Third, it has been observed that apoE is secreted in association with radiolabeled cholesterol and phospholipid from C127 cells and mouse peritoneal macrophages (14, 15). In the latter cell model, however, it is not possible to determine whether macrophage apoE secretion and cholesterol secretion are causally related or simply associated processes.

In order, therefore, to directly investigate the impact of apoE secretion on macrophage cholesterol balance, we have elected to study the J774 macrophage cell (which does not express its endogenous apoE gene) that has been transfected to express a human apoE cDNA under the control of the human metallothionine IIA promoter. We have previously characterized the kinetics of apoE synthesis and secretion from these cells (16). The use of this model has a number of advantages for delineating the influence of apoE secretion on macrophage cholesterol balance. The cholesterol metabolism of J774 cells has been extensively characterized (11, 12, 17). Further, this model offers the opportunity to investigate apoE modulation of cholesterol balance without the potentially confounding reciprocal modulation of apoE synthesis by cholesterol balance because the metallothionine IIA promoter does not respond to cholesterol loading (16). Most importantly, we can directly compare cholesterol balance in transfected apoE expressing and control non-expressing cells after experimental interventions known to modulate macrophage cholesterol homeostasis (11, 12, 16), thereby determining the precise contribution of apoE secretion to this process. A similar experimental approach has been used to study the influence of apoE synthesis on the cholesterol metabolism of steroidogenic cells (18). In steroidogenic cells, expression of apoE may be involved in modulating cholesterol utilization for steroid hormone production. In this report, we demonstrate that macrophage expression of apoE enhances cholesterol efflux from cholesterol-loaded macrophages to extracellular cholesterol acceptors.

METHODS

Materials

[^{3}H]cholesterol (46 Ci/mmol) was purchased from Amersham Corporation. S58035 was obtained from Sandoz Pharmaceuticals. Cyclic AMP was used as its 8-(4-chlorophenyithiol) derivative and was obtained from Sigma. All other materials were from previously described sources (5–7, 16).

Lipoproteins and lipid dispersions

Human LDL (d 1.019–1.063 g/ml) and HDL_3 (d 1.125–1.210 g/ml) were prepared by ultracentrifugation in KBr as previously described (7). The HDL_3 used in these experiments had no apoE detectable by Coomassie blue staining of SDS-PAGE gels. LDL was acetylated by the repeated addition of acetic anhydride (7). All lipoprotein concentrations are given in terms of protein content. Lipid dispersions with a free cholesterol-phosphatidylcholine molar ratio > 2 were prepared as described (19). Briefly, cholesterol:phosphatidylcholine mixtures were sonicated for 10 min, twice, and these dispersions were recovered in the supernatant after a 30-min spin at 45,000 *g*. The dispersions were suspended in cell culture media at 225 μg/ml of cholesterol and radiolabeled cholesterol was added such that its final concentration during incubation with the cells would be 1–2.5 μCi/ml. This medium was then filter-sterilized and incubated for 48 h at 37°C prior to use.

Cell culture

Stably transfected J774 cells were prepared by cotransfection of the apoE cDNA expression vector containing the human metallothionine IIA promoter with pSV_2 neo by calcium phosphate co-precipitation in the presence of 10 mM ammonium chloride as previously described (16). All experiments were done with clonally derived apoE-secreting cells. Metal ions did not further induce apoE expression from these cells and was not used in our experiments. All experiments were performed using the J774E-1 line unless otherwise indicated. Cells transfected with pSV_2 neo alone (neo-J774) did not synthesize or secrete detectable apoE and were used in these experiments as control non-secreting cells. These control cells were derived from a mixed mass culture from > 50 separate colonies after G418 (Geneticin, GIBCO-BRL) selection. Cells were selected and maintained in G418 (400 μg/ml) until 2 weeks prior to initiation of experiments. After plating identical numbers of apoE-secreting and non-secreting control cells, there were no differences in cell protein content at the end of experimental incubations.

For all experiments in which cellular cholesterol mass was measured, 1.2×10^6 cells were plated in 10% FBS in DMEM in 35-mm wells. After 72 h, fresh medium of the same composition containing 50–100 μg/ml of ALDL was added for an additional 24–48 h. At that time, cells were washed with DMEM and placed in 0.2% BSA/DMEM for an additional 24 h to allow for equilibration, at which time the experimental incubations described in the figure and table legends were begun. Over the course of these experiments cells subjected to identical incubations, but without ALDL, contained 18.1–21.4 μg/mg free cholesterol and 0–1.5 μg/mg cholesterol ester. For experiments in which ^{3}H radioactivity in cholesterol was analyzed, identical incubations were carried out except that the loading medium contained 50 μg/ml ALDL and 225 μg/ml free cholesterol in lipid dispersions containing a final concentration of 1–2.5 μCi/ml [^{3}H]cholesterol. This labeling medium was incubated with cells for 48 h. At that time, cells were washed and placed in 0.2% BSA/DMEM for 24 h to allow equilibration of label. Experimental incubations described in the figure legends were then begun. During the 24-h period of experimental incubations, apoE-expressing cells secreted 915 ng of apoE into serum-free culture medium as measured by ELISA assay.

Lipid analyses

Cellular free and total cholesterol (after saponification) were measured in hexane–isopropyl alcohol extracts. Extracts were analyzed by gas–liquid chromatography using coprostanol as an internal standard as previously described (7). [^{3}H]cholesterol was measured in Bligh-Dyer extracts of cell culture medium and [^{3}H]cholesterol and cholesteryl ester were measured in hexane–isopropyl alcohol extracts of cells (20). These extracts were analyzed by thin-layer chromatography on silica gel G developed with petroleum ether–ethyl ether–acetic acid 75:25:1. Appropriate spots, visualized in iodine vapor, were scraped into scintillant for counting. The amount of labeled cholesterol secreted was expressed as the fraction of [^{3}H]cholesterol counts in the medium divided by the total number (medium and cells) of ^{3}H radioactivity in cholesterol and cholesterol ester.

Other

Cellular protein was measured in NaOH extracts by the method of Lowry et al. (21) using BSA as standard. Statistical analyses were performed using a two-tailed *t*-test.

RESULTS

In order to examine the effect of apoE secretion on cholesterol efflux from cholesterol-loaded J774 cells, we first determined whether cholesterol loading of cells via the scavenger receptor using ALDL was altered by apoE secretion. As shown in **Figure 1,** there is no significant difference in the total mass of cellular cholesterol in J774 E1 versus neo-J774 cells immediately after incubation with ALDL (0 h) or after an additional 24 h in serum-free medium (24 h). Therefore, using these loading conditions, apoE synthesis did not influence the efficiency of loading of cells with cholesterol derived from ALDL. This similarity at 0 h allowed us to compare cholesterol mass

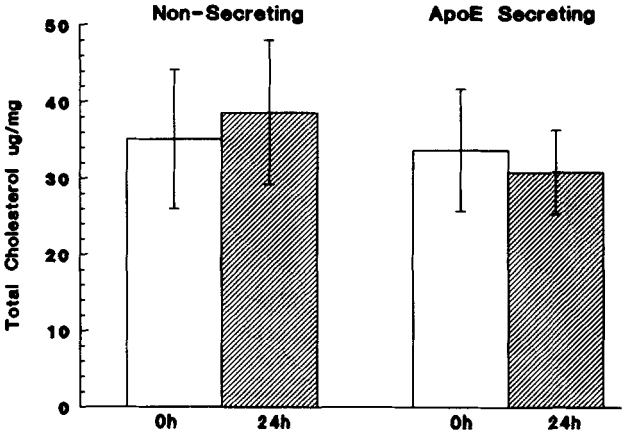

Fig. 1. Total cellular cholesterol mass in apoE-secreting and non-secreting J774 cells in serum-free medium. Cells were plated and incubated with ALDL as described in Methods. After a 24-h incubation period in 0.2% BSA/DMEM, the cultures were washed and placed in fresh 0.2% BSA/DMEM and harvested immediately (open bars) or after an additional 24 h (hatched bars). Total cellular cholesterol per mg cell protein was determined as described in Methods. The values shown represent the mean and standard deviation from 18 replicate cultures. Differences are not significant at the $P < 0.05$ level.

in these cells after an additional 24 h of experimental interventions and interpret the difference in cholesterol mass as net efflux. The similarity in cholesterol mass after 24 h in 0.2% BSA, therefore, suggests that there is no difference in cholesterol efflux between apoE-secreting and non-secreting cells under serum-free conditions, i.e., in the absence of an extracellular acceptor of cholesterol. The lack of efflux from apoE-secreting J774 cells under these conditions is similar to what is observed in primary cultures of apoE-secreting macrophages which also do not lose measurable cholesterol mass in the absence of an extracellular acceptor of cholesterol (10).

This result was confirmed using a potentially more sensitive approach wherein cellular cholesterol is labeled to equilibrium. For these experiments, we loaded cells by incubation with ALDL and lecithin/cholesterol vesicles containing [^{3}H]cholesterol. After labeling and a 24-h equilibration period, we measured the free cholesterol that was lost to the medium over a subsequent 24-h incubation from apoE-secreting or non-secreting cells. As shown in **Figure 2A,** in three separate experiments, there was no difference in the amount of cholesterol lost to serum-free medium between these cell lines; confirming the results of the experiment shown in Fig. 1. The data in Figs. 2B and 2C, however, indicate that the addition of two drugs (cyclic AMP or S58035), which have a common effect of shifting cellular cholesteryl ester-free cholesterol equilibrium in favor of free cholesterol, did produce enhanced efflux from J774 E1 cells compared to neo-J774 cells. These results indicated that apoE secretion could enhance cholesterol efflux in the presence of these agents even in the absence of an extracellular acceptor for cholesterol. However, in most experiments, this enhancement was small and a significant effect on cellular cholesterol mass could not be measured between J774 E1 and neo-J774 cells incubated with these same drugs (not

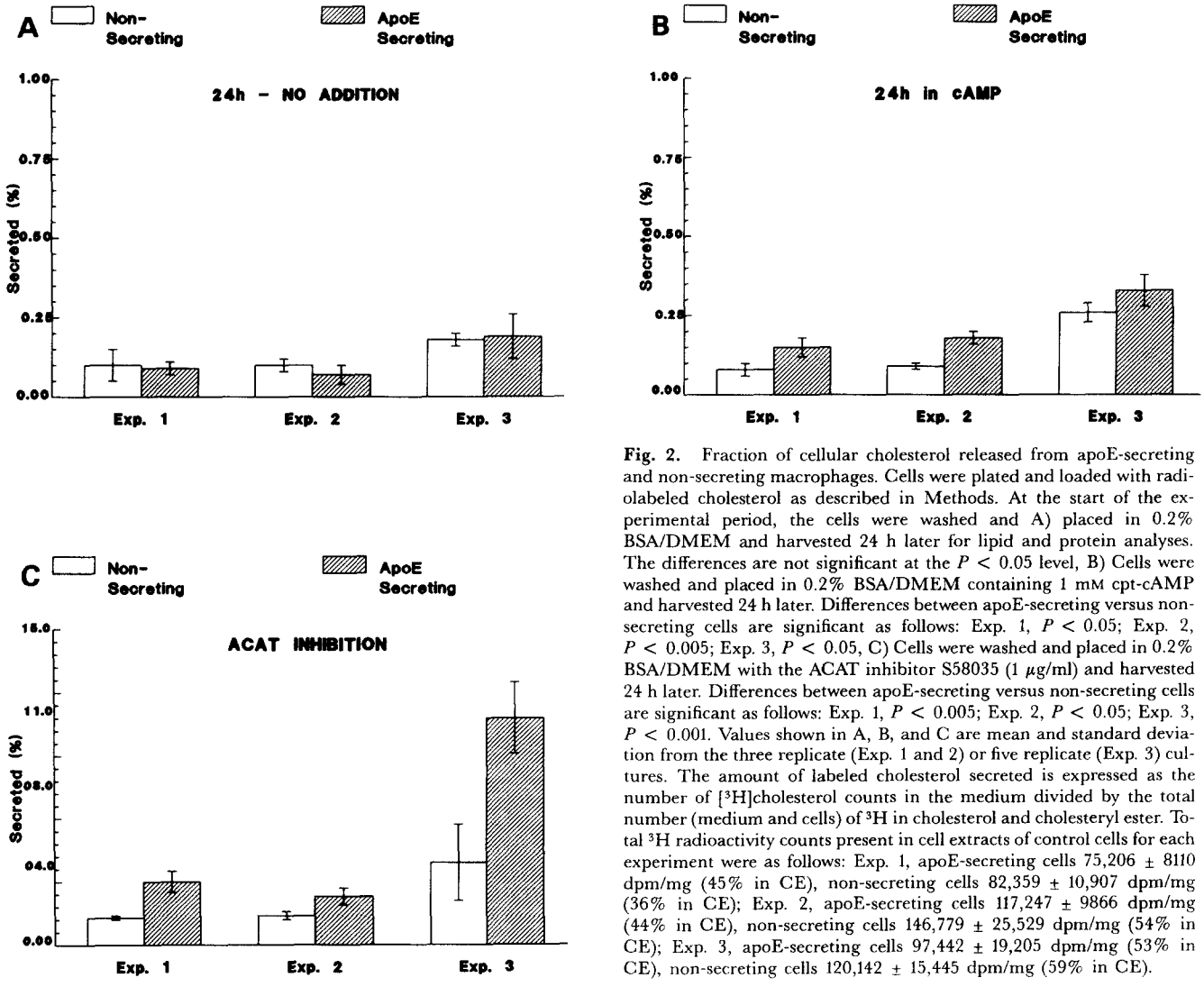

Fig. 2. Fraction of cellular cholesterol released from apoE-secreting and non-secreting macrophages. Cells were plated and loaded with radiolabeled cholesterol as described in Methods. At the start of the experimental period, the cells were washed and A) placed in 0.2% BSA/DMEM and harvested 24 h later for lipid and protein analyses. The differences are not significant at the $P < 0.05$ level, B) Cells were washed and placed in 0.2% BSA/DMEM containing 1 mM cpt-cAMP and harvested 24 h later. Differences between apoE-secreting versus non-secreting cells are significant as follows: Exp. 1, $P < 0.05$; Exp. 2, $P < 0.005$; Exp. 3, $P < 0.05$, C) Cells were washed and placed in 0.2% BSA/DMEM with the ACAT inhibitor S58035 (1 μg/ml) and harvested 24 h later. Differences between apoE-secreting versus non-secreting cells are significant as follows: Exp. 1, $P < 0.005$; Exp. 2, $P < 0.05$; Exp. 3, $P < 0.001$. Values shown in A, B, and C are mean and standard deviation from the three replicate (Exp. 1 and 2) or five replicate (Exp. 3) cultures. The amount of labeled cholesterol secreted is expressed as the number of [^{3}H]cholesterol counts in the medium divided by the total number (medium and cells) of ^{3}H in cholesterol and cholesteryl ester. Total ^{3}H radioactivity counts present in cell extracts of control cells for each experiment were as follows: Exp. 1, apoE-secreting cells 75,206 ± 8110 dpm/mg (45% in CE), non-secreting cells 82,359 ± 10,907 dpm/mg (36% in CE); Exp. 2, apoE-secreting cells 117,247 ± 9866 dpm/mg (44% in CE), non-secreting cells 146,779 ± 25,529 dpm/mg (54% in CE); Exp. 3, apoE-secreting cells 97,442 ± 19,205 dpm/mg (53% in CE), non-secreting cells 120,142 ± 15,445 dpm/mg (59% in CE).

TABLE 1. Effect of HDL_3 on cellular cholesteryl ester and free cholesterol mass compared to a 24-h incubation in serum-free medium

	Free Cholesterol		Cholesteryl Ester	
Treatment	ApoE-Secreting	Non-Secreting	ApoE-Secreting	Non-Secreting
	μg/mg		*μg/mg*	
Serum-free medium	19.2 ± 1.4	18.7 ± 1.3	10.5 ± 3.9	12.8 ± 2.8
HDL_3	18.5 ± 0.8	18.9 ± 1.0	3.2 ± 2.0[a]	8.5 ± 4.1[b]

Cholesterol-loaded J774 cells were prepared as described in Methods. At the start of the experimental period, cells were washed and placed in serum-free medium alone or with HDL_3 (400 μg/ml). After 24 h, cells were harvested for lipid and protein analyses. Values shown are mean ± SD from five replicate cultures.

[a] $P < 0.01$, serum-free versus HDL_3.

[b] P, not significant, serum-free versus HDL_3.

shown). We, therefore, performed a series of experiments using human HDL_3, a physiologic cholesterol acceptor likely to be present in the pericellular space. As noted above, primary cultures of apoE-secreting macrophages efflux cholesterol most efficiently in the presence of an extracellular acceptor of cholesterol. For these experiments, the decrease in cellular cholesterol produced by HDL_3 was taken as net efflux.

Previous studies have shown that HDL_3 does not stimulate cholesterol efflux from wild-type J774 cells (12). To determine whether apoE secretion alters this response, we incubated cholesterol-loaded J774 E1 and neo-J774 cells with HDL_3 and measured its effect on cellular cholesterol content compared to an incubation in serum-free medium alone (**Table 1**). As shown, after a 24-h incubation, the cholesteryl ester mass of J774 E1 cells was 10.5 ± 3.9 in serum-free medium compared to 3.2 ± 2.0 μg/mg in the presence of HDL_3, a decrease of 7.3 μg/mg ($P < 0.01$). In non-secreting cells, HDL_3 incubation did not significantly alter cellular cholesteryl ester levels, consistent with previous observations (12, 16). Free cholesterol levels did not change significantly in either cell type.

In a separate experiment, we evaluated the change in cholesterol over a 24-h period with HDL_3 in J774 E1 and neo-J774 cells. The cells were loaded with cholesterol as described in Methods. We harvested half of the cultures immediately prior to adding HDL_3 (0 h) and the balance of the cultures were harvested after a 24-h incubation in HDL_3. These results are shown in **Table 2.** At 0 h, prior to the HDL_3 incubation, there is no significant difference between apoE-secreting and non-secreting cells in free cholesterol or cholesteryl ester mass. After 24 h in HDL_3, cholesteryl ester mass decreased by 5.6 μg/mg to barely detectable levels in the J774 E1 cells while there was very little change (1.6 μg/mg) in cholesterol ester mass in neo-J774 cells. The results of these two lines of experimentation, therefore, indicated that apoE secretion in the J774 E1 line is associated with significant net cholesterol efflux in the presence of HDL_3.

Given the dependence of effective efflux on the availability of free cholesterol, we next evaluated the possibility that apoE directly altered free cholesterol–cholesteryl ester balance, to make free cholesterol more available, and thereby facilitated efflux to HDL_3. As noted above, the balance between free cholesterol and cholesteryl ester is modulated by the activity of two enzymes, cytoplasmic neutral cholesterol ester hydrolase and ACAT. We therefore evaluated the potential influence of apoE on these

TABLE 2. Decrease in cellular cholesterol during a 24-h incubation in HDL_3

	Free Cholesterol		Cholesteryl Ester	
Treatment	ApoE-Secreting	Non-Secreting	ApoE-Secreting	Non-Secreting
	μg/mg		*μg/mg*	
0 h	20.4 ± 2.0	22.3 ± 2.7	6.4 ± 2.4	9.3 ± 2.2
24 h with HDL_3	18.7 ± 1.9	25.1 ± 0.3[a]	0.8 ± 0.7	7.7 ± 1.9[a]

Cholesterol-loaded cells were prepared as described in Methods. At the start of the experimental period, cells were washed and harvested immediately (0 h) or after an additional 24 h with 400 μg/ml HDL_3. Values shown are the mean ± SD of triplicate cultures. Values at 0 h are not significantly different between apoE-secreting and non-secreting cells.

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

TABLE 3. Effect of apoE secretion on the hydrolysis of cholesterol ester

Treatment	Radioactivity in CE	
	ApoE Secreting	Non-Secreting
	dpm/mg	
0 h	62,919 ± 5,270	57,968 ± 1,356
24 h	33,821 ± 5,159	38,382 ± 6,114
24 h with S58035	9,278 ± 1,309	9,615 ± 712

J774 cells were plated and loaded with [^{3}H]cholesterol as described in Methods. After a 24-h equilibration period in serum-free medium, cells were harvested (0 h) or cultured for another 24 h in serum-free medium alone (24 h) or this medium plus 1 μg/ml of S58035. Radioactivity was measured in cholesteryl ester isolated from hexane–isopropanol cell extracts by thin-layer chromatography. Values shown are mean ± SD from triplicate cultures except for the non-secreting 0 h value which represents the mean of duplicates. There are no significant differences between apoE-secreting and non-secreting cells at any time point.

pathways. To compare the hydrolysis of cholesteryl ester between J774 E1 and neo-J774 cells in the absence of re-esterification, the cells were enriched with radiolabeled cholesterol and then incubated for an additional 24 h in serum-free medium alone or this medium + S58035 at 1 μg/ml. In preliminary experiments we showed that this concentration of S58035 inhibited > 95% of the incorporation of [^{14}C]oleic acid into cholesteryl ester (not shown). The results of this experiment are shown in **Table 3.** There was no significant difference in cholesteryl ester radioactivity between J774 E1 and neo-J774 cells at 0 h or after a 24-h incubation in the presence or absence of S58035. This result therefore suggests that apoE synthesis does not influence the rate of cholesteryl ester hydrolysis in these cells. However, radioactivity in cholesteryl ester did decrease to a slightly greater degree (62,919 to 9278 dpm/mg) in J774 E1 cells than in neo-J774 cells (57,988 to 9615 dpm/mg) suggesting that a subtle difference in ester hydrolysis may not have been detected.

A potential role for this in efflux was, therefore, further examined by measuring cellular cholesterol mass after incubation with HDL_3 and cAMP/S58035. If apoE secretion enhanced efflux by altering cholesteryl ester-free cholesterol equilibrium, the addition of S58035 (to inhibit ACAT) with cAMP (to stimulate cholesterol ester hydrolysis) should diminish or perhaps even abolish the difference in efflux between J774 E1 and neo-J774 cells in the presence of HDL_3. As shown in **Table 4,** addition of both agents with HDL_3 leads to an almost complete loss of cholesteryl ester mass in both cell types; from 19.5 ± 4.1 to 1.6 ± 1.4 μg/mg in J774 E1 cells and from 18.0 ± 5.1 to 0.8 ± 1.4 μg/mg in neo-J774 cells. However, for the neo-J774 cells, the loss of CE was quantitatively accounted for by an increase in free cholesterol, while in J774 E1 cells free cholesterol did not substantially change due to cholesterol efflux. This is reflected in the total cholesterol which remained unchanged in the neo-J774 cells (42.1 ± 5.8 to 43.4 ± 6.8 μg/mg) but substantially decreased in the J774 E1 cells (41.4 ± 4.4 vs. 24.4 ± 0.9 μg/mg) after incubation with cAMP + S58035 + HDL_3.

As noted above, the neo-J774 cell line was derived from a pool > 50 stably transfected J774 colonies. The J774 E1 line was clonally derived from a single cell. We therefore evaluated two other clonally derived apoE-secreting J774 cell lines to establish that the enhanced cholesterol efflux observed in J774 E1 cells was a function of apoE secretion. These data are shown in **Table 5.** All apoE-secreting J774 lines displayed a significant decrease in cholesteryl ester mass during incubation with HDL_3. The decrease in total cellular cholesterol in response to HDL_3 reflected the cholesteryl ester change for each of the cell lines shown and is as follows; non-secreting control J774 cells, 0.4; J774 E1 cells, 5.7; J774 E2, 12.3; J774 E3, 18.7 μg/mg cell protein.

DISCUSSION

In this paper we have demonstrated that the secretion of human apoE by J774 cells facilitates HDL_3-mediated cholesterol efflux compared to control non-expressing cells. While our data do not exclude a subtle effect of apoE

TABLE 4. Comparison of cellular cholesterol response to HDL_3 after conversion of esterified cholesterol to free cholesterol using cAMP + S58035

Treatment	Free Cholesterol		Cholesteryl Ester		Total Cholesterol	
	ApoE-Secreting	Non-Secreting	ApoE-Secreting	Non-Secreting	ApoE-Secreting	Non-Secreting
	μg/mg		*μg/mg*		*μg/mg*	
0 h	21.9 ± 0.7	24.1 ± 1.7	19.5 ± 4.1	18.0 ± 5.1	41.4 ± 4.4	42.1 ± 5.8
24 h (S58035 + cAMP + HDL_3)	22.8 ± 1.4	42.5 ± 3.3[a]	1.6 ± 1.4	0.8 ± 1.4	24.4 ± 0.9	43.3 ± 6.8[a]

Cells were loaded with cholesterol as described in Methods. At the start of the experimental period, cells were harvested (0 h) or washed and incubated for an additional 24 h in HDL_3 (400 μg/ml), cAMP (1 mM), and S58035 (1 μg/ml). Cells were then harvested for lipid and protein analyses. Values shown are mean ± SD from quadruplicate cultures.

[a] $P < 0.002$ apoE-secreting versus non-secreting.

TABLE 5. Demonstration of enhanced cholesterol efflux to HDL_3 using additional apoE-secreting clonal lines

	Cholesteryl Ester		
	Control	HDL_3	Difference
		μg/mg	
Non-secreting	28.1 ± 2.2	27.7 ± 4.2	NS
J774 E-1	19.6 ± 1.6	15.6 ± 1.6	$P < 0.05$
J774 E-2	50.7 ± 1.9	41.9 ± 3.7	$P < 0.05$
J774 E-3	46.4 ± 4.9	31.3 ± 2.1	$P < 0.02$

J774 cells from a transfected non-secreting pool or from three apoE-secreting lines (J774 E-1, J774 E-2, J774 E-3) clonally derived from single cells were prepared and loaded with cholesterol as described in Methods. At the start of the experimental period, cells were washed and placed in serum-free medium (control) or this medium plus HDL_3. After 24 h cells were harvested for protein and lipid analyses. Values shown are mean ± SD from quadruplicate cultures. NS, not significantly different. Differences for total cholesterol mass are given in the text.

expression on the rate of cholesteryl ester hydrolysis, the enhancement of efflux by apoE secretion is not likely to be due to alteration of free cholesterol–cholesteryl ester equilibrium as agents that increase the availability of free cholesterol in J774 cells do not abolish the difference in HDL_3-mediated efflux between apoE-secreting and non-secreting cells (Table 4). Therefore, inhibition of cholesterol esterification or acceleration of cholesteryl ester hydrolysis is unlikely to account for the enhanced efflux to HDL_3. The transfected J774 E1 cells used for these studies secreted 0.92 μg/mg per 24 h of apoE into serum-free medium. This amount is similar to what has been reported for apoE secretion from primary cultures of human monocyte-derived macrophages, which ranged from 0.64 to 2.82 μg/mg per 24 h (22). The data in Fig. 1 and 2A suggest that secretion of a physiologic amount of apoE, in the absence of an extracellular cholesterol acceptor, is not by itself sufficient to enhance cholesterol efflux from cholesterol-loaded J774 cells. However, drugs that expand the cellular free cholesterol pool facilitate secretion of radiolabeled cholesterol by the apoE expressing cells; even in the absence of HDL_3 (Figs. 2B and 2C). In the absence of HDL_3 this effect is small, however, as there were no measurable differences in total cholesterol mass under these conditions.

The data from Table 4 indicate that a substantial mass of cholesterol (17 μg of sterol per mg cell protein) can be cleared from apoE-secreting J774 cells in the presence of HDL_3, cAMP, and S58035. The human metallothionine IIA promoter used to drive apoE expression in our expression vector contains cAMP-responsive elements and we have measured a 2-fold increase of apoE mRNA and apoE secretion in the apoE transfected cells after incubation in cAMP (not shown). If this enhancement of apoE secretion contributes to the substantial efflux of cholesterol to HDL_3 in the presence of cAMP, it would serve to underscore the importance of apoE for cholesterol efflux. Based on observations made in non-apoE-secreting cells, however, we believe it is likely that cAMP facilitates efflux, even in apoE-secreting cells, by non-apoE-dependent mechanisms. Others have, in fact, suggested that second messenger systems including those involving cAMP might be involved in cellular cholesterol transport (23).

If apoE does not enhance efflux by perturbing free cholesterol–cholesteryl ester balance, what other mechanisms could be considered? First, apoE could modulate second messenger pathways in macrophages as has been shown in steroidogenic cells (24, 25). As noted above, these second messenger pathways may regulate cellular cholesterol transport and increase the movement of free cholesterol to the plasma membrane, as suggested by data obtained in human skin fibroblasts and bovine aortic endothelial cells (23). Hokland et al. (26) have recently shown that addition of cAMP to cholesterol-loaded human skin fibroblasts increases the proportion of sterol that is accessible to exogenous cholesterol oxidase and stimulates the efflux of intracellular sterols. While it is possible that apoE alters the subcellular distribution of free cholesterol and thereby enhances efflux, this is unlikely to result from activation of endogenous cAMP by apoE. Our data indicate that in the presence of CPT-cAMP at 1 mM, a concentration which maximally stimulates cholesteryl ester hydrolysis in J774 cells (12), the difference in efflux between J774 E1 and neo-J774 cells is actually widened and not narrowed as one would predict if apoE enhanced efflux via a mechanism that involved activating endogenous cAMP. Alternatively, apoE secretion could directly interact with cellular lipids to alter cellular cholesterol transport or enhance cholesterol desorption from plasma membranes. Consistent with the latter proposal, data from Leblond and Marcel (27) and from Lilly-Stauderman et al. (28) have indicated that endogenously synthesized apoE remains associated with the cell surface in HepG2 cells. One could therefore speculate that endogenously produced apoE could remain associated with plasma membrane and facilitate lipid movement to exogenously added HDL_3. This process could, for example, involve enhancing the formation of cholesterol-enriched domains of the plasma membrane given the importance of plasma membrane lipid composition for modulating cholesterol efflux kinetics from intact cells (29). Plasma membrane-associated apoE could also enhance efflux by facilitating the interaction of HDL_3 with cholesterol-rich plasma membrane. Alternatively, secreted apoE could interact with HDL_3 to enhance its ability to promote efflux. The data from Figs. 2B and 2C, however, suggest that apoE secretion can stimulate cholesterol efflux from macrophages by a mechanism independent of HDL_3.

Finally, the observation that endogenously synthesized and secreted apoE modulates macrophage cholesterol balance needs to be viewed in the context of what is known

regarding regulation of macrophage apoE expression. In several macrophage cell types, the process of differentiation from a monocytic to macrophage phenotype is accompanied by the increased capacity to synthesize and secrete apoE, just as it is accompanied by the increased capacity to ingest (via the scavenger receptor) and store large amounts of cholesterol (30). Further, in fully differentiated macrophages, apoE synthesis and secretion is regulated by small changes in macrophage free cholesterol content at the transcriptional stage and by the presence of extracellular cholesterol acceptors at a post-translational site (5–7, 16). The reciprocal modulation of apoE by cholesterol, and of cholesterol by apoE, in the macrophage suggests a regulatory loop important in cholesterol homeostasis. Such a loop could function to protect macrophage membranes from the toxic effects of excess free cholesterol. Alternatively, this loop could function to allow macrophages to more effectively convert extracellular cholesterol from a toxic and/or unusable form (i.e., within modified/oxidized lipoproteins or degenerating cells) to a form that is rendered nontoxic and is either usable in surrounding tissues for membrane synthesis or more efficiently returned to the liver.

Supported by grants HL39653 and HL15062 from the National Institutes of Health. Dr. Mazzone is an Established Investigator of the American Heart Association. This work was done during the tenure of a Grant-In-Aid Award from the American Heart Association and Parke-Davis (to TM). The authors would like to thank Dr. Elaine Krul for performing the apoE ELISA assay, Dr. Godfrey Getz for helpful discussions regarding this work, Lydia Blachowicz and Madhuri Krishna for technical assistance, and Beverly Burge for preparing the manuscript.

Manuscript received 12 August 1993, in revised form 13 January 1994, and in re-revised form 4 March 1994.

REFERENCES

1. Basu, S. K., M. S. Brown, Y. K. Ho, R. J. Havel, and J. L. Goldstein. 1981. Mouse macrophages synthesize and secrete a protein resembling apolipoprotein E. *Proc. Natl. Acad. Sci. USA.* **78:** 11657–11662.
2. Newman, T. C., P. A. Dawson, L. L. Rudel, and D. L. Williams. 1985. Quantitation of apolipoprotein E mRNA in the liver and peripheral tissues of nonhuman primates. *J. Biol. Chem.* **260:** 2452–2457.
3. Driscoll, D. M., and G. S. Getz. 1984. Extrahepatic synthesis of apolipoprotein E. *J. Lipid Res.* **25:** 1368–1379.
4. Mahley, R. W. 1988. Apolipoprotein E: cholesterol transport protein with expanding role in cell biology. *Science.* **240:** 622–630.
5. Mazzone, T., H. Gump, P. Diller, and G. S. Getz. 1987. Macrophage free cholesterol content regulates apolipoprotein E synthesis. *J. Biol. Chem.* **262:** 11657–11662.
6. Mazzone, T., K. Basheeruddin, and C. Poulos. 1989. Regulation of macrophage apolipoprotein E gene expression by cholesterol. *J. Lipid Res.* **30:** 1055–1064.
7. Mazzone, T., and K. Basheeruddin. 1991. Dissociated regulation of macrophage low density lipoprotein receptor and apolipoprotein E gene expression by sterol. *J. Lipid Res.* **32:** 507–514.
8. Schwartz, C. J., A. J. Valente, E. A. Sprague, J. L. Kelley, C. A. Suenram, D. T. Graves, M. M. Rozek, E. H. Edwards, and R. Delgado. 1986. Monocyte-macrophage participation in atherogenesis: inflammatory components of pathogenesis. *Semin. Thromb. Hemostasis.* **12:** 79–86.
9. Ross, R. 1993. The pathogenesis of atherosclerosis: a perspective for the 1990s. *Nature.* **362:** 801–809.
10. Brown, M. S., Y. K. Ho, and J. L. Goldstein. 1980. The cholesteryl ester cycle in macrophage foam cells. *J. Biol. Chem.* **255:** 9344–9352.
11. Khoo, J. C., E. M. Mahoney, and D. Steinberg. 1981. Neutral cholesterol esterase activity in macrophages and its enhancement by cAMP-dependent protein kinase. *J. Biol. Chem.* **256:** 12659–12661.
12. Bernard, D. W., A. Rodriguez, G. H. Rothblat, and J. M. Glick. 1991. cAMP stimulates cholesteryl ester clearance to high density lipoproteins in J774 macrophages. *J. Biol. Chem.* **266:** 710–716.
13. Hara, H., and S. Yokoyama. 1991. Interaction of free apolipoproteins with macrophages: formation of high density lipoprotein-like lipoproteins and reduction of cellular cholesterol. *J. Biol. Chem.* **266:** 3080–3086.
14. Herscovitz, H., D. Gantz, A. M. Tercyak, V. I. Zannis, and D. M. Small. 1992. Expression of human apolipoprotein E but not that of apolipoprotein A-I by mouse C127 cells is associated with increased secretion of lipids in the form of vesicles and discs. *J. Lipid Res.* **33:** 791–803.
15. Dory, L. 1989. Synthesis and secretion of apoE in thioglycolate-elicited mouse peritoneal macrophages: effect of cholesterol efflux. *J. Lipid Res.* **30:** 809–816.
16. Mazzone, T., L. Pustelnikas, and C. A. Reardon. 1992. Post-translational regulation of macrophage apoprotein E production. *J. Biol. Chem.* **267:** 1081–1087.
17. Tabas, I., W. J. Rosoff, and G. C. Boykow. 1988. Acyl coenzyme A:cholesterol acyl transferase in macrophages utilizes a cellular pool of cholesterol oxidase-accessible cholesterol as substrate. *J. Biol. Chem.* **263:** 1266–1272.
18. Reyland, M. E., J. T. Gwynne, P. Forgez, M. M. Prack, and D. L. Williams. 1991. Expression of the human apolipoprotein E gene suppresses steroidogenesis in mouse Y1 adrenal cells. *Proc. Natl. Acad. Sci. USA.* **88:** 2375–2379.
19. Glick, J. M., S. J. Adelman, M. C. Phillips, and G. H. Rothblat. 1983. Cellular cholesteryl ester clearance. *J. Biol. Chem.* **25:** 13423–13450.
20. Hirsch, L. J., and T. Mazzone. 1986. Dexamethasone modulates lipoprotein metabolism in cultured human monocyte-derived macrophages. *J. Clin. Invest.* **77:** 485–490.
21. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193:** 265–275.
22. Wang-Iverson, P., J. C. Gibson, and W. V. Brown. 1985. Plasma apolipoprotein secretion by human monocyte-derived macrophages. *Biochim. Biophys. Acta.* **834:** 256–262.
23. Mendez, A. J., J. F. Oram, and E. L. Bierman. 1991. Protein kinase C as a mediator of high density lipoprotein receptor-dependent efflux of intracellular cholesterol. *J. Biol. Chem.* **266:** 10104–10111.
24. Reyland, M. E., and D. L. Williams. 1991. Suppression of cAMP-mediated signal transduction in mouse adrenocortical cells which express apolipoprotein E. *J. Biol. Chem.* **266:** 21099–21104.
25. Reyland, M. E., M. M. Prack, and D. L. Williams. 1992. Elevated levels of protein kinase C in Y1 cells which express apolipoprotein E decrease basal steroidogenesis by inhibiting

expression of P450-cholesterol side chain cleavage mRNA. *J. Biol. Chem.* **267:** 17933–17938.
26. Hokland, B. M., J. P. Slotte, E. L. Bierman, and J. F. Oram. 1993. Cyclic AMP stimulates efflux of intercellular sterol from cholesterol loaded cells. *J. Biol. Chem.* **268:** 25343–25349.
27. Leblond, L., and Y. L. Marcel. 1993. Uptake of high density lipoprotein cholesterol ester by HepG2 cells involves apolipoprotein E localized on the cell surface. *J. Biol. Chem.* **268:** 1670–1676.
28. Lilly-Stauderman, M., T. L. Brown, A. Balasubramaniam, and J. A. K. Harmony. 1993. Heparin releases newly synthesized cell surface-associated apolipoprotein E from HepG2 cells. *J. Lipid Res.* **34:** 190–200.
29. Bellini, F., M. C. Phillips, C. Pickell, and G. H. Rothblat. 1984. Role of the plasma membrane in the mechanism of cholesterol efflux from cells. *Biochim. Biophys. Acta.* **777:** 209–215.
30. Basheeruddin, K., C. Rechtoris, and T. Mazzone. 1992. Transcriptional and post-transcriptional control of apolipoprotein E gene expression in differentiating human monocytes. *J. Biol. Chem.* **267:** 1219–1224.