Apolipoprotein E-dependent cholesterol efflux from macrophages: kinetic study and divergent mechanisms for endogenous versus exogenous apolipoprotein E

Chen-Yi Lin, Hongwei Duan, and Theodore Mazzone[1]

Departments of Medicine and Biochemistry, Rush Medical College, 1653 W. Congress Parkway, Chicago, IL 60612

Abstract **In these studies, we have utilized a J774 macrophage model in order to compare phospholipid and cholesterol efflux kinetics in macrophage cells that do not express endogenous apoE to cells transfected to express physiologic levels of human apoE. This model was also used to compare the effect of exogenously added versus endogenously expressed apoE on cholesterol efflux kinetics from macrophages. ApoE expression increased free cholesterol and phospholipid efflux into the medium, but did not change the free cholesterol/phospholipid molar ratio of secreted lipids. Kinetic examination showed that free cholesterol and phospholipid appeared simultaneously in the medium, and that cholesterol loading widened the difference in the rate of cholesterol efflux between apoE-expressing and non-expressing macrophages. Addition of exogenous lipid-free apoE added to non-expressing cells, at a >2-fold higher medium concentration than that produced by endogenous expression, produced less cholesterol efflux than that observed from apoE-expressing cells. The addition of phosphatidylcholine liposomes substantially increased cholesterol efflux from apoE-expressing and non-expressing J774 cells. Addition of these liposomes eliminated the enhanced cholesterol efflux produced by addition of exogenous apoE. On the other hand, even in the presence of phosphatidylcholine liposomes, cholesterol efflux rates remained significantly higher from apoE-expressing macrophages than non-expressing cells. Similar results were obtained when efflux was studied in the presence of cyclodextrin. These results suggest that endogenous expression of apoE by macrophages alters cell cholesterol balance via mechanisms distinct from those utilized by the extracellular addition of apoE, and may involve intracellular or pericellular mechanisms.**—Lin, C-Y., H. Duan, and T. Mazzone. **Apolipoprotein E-dependent cholesterol efflux from macrophages: kinetic study and divergent mechanisms for endogenous versus exogenous apolipoprotein E.** ***J. Lipid Res.*** **1999.** 40: **1618–1626.**

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Human atherosclerotic lesions contain abundant apoE usually found in association with macrophages which are the major source of apoE at this site (1). Expression of apoE by macrophages has been found to have potent anti-atherogenic properties (2–6). In apoE-deficient mice, macrophage-specific expression of apoE can markedly attenuate development of atherosclerotic lesions, even in the presence of high levels of circulating atherogenic lipoproteins (5). Conversely, elimination of apoE expression specifically in macrophages has been shown to dramatically increase the vulnerability of the vessel wall to atherogenic insult (6).

Multiple properties of apoE need to be considered in evaluating mechanisms for its anti-atherogenic effect(s) in the vessel wall (7). Modulation of platelet, arterial smooth muscle cell, and lymphocyte function by apoE have all been demonstrated (7–11). However, a major aspect of the anti-atherogenic effect of apoE is likely to relate to its promotion of cellular cholesterol efflux. It has been known for many years that addition of exogenous lipid-free apolipoproteins, including apoE, to several cell types will promote removal of cellular cholesterol, and hypotheses have been put forward regarding the potential mechanism(s) for such efflux (7, 12–15). Recently, it has also been documented that expression of endogenous apoE by macrophages enhances cholesterol efflux from macrophages (16–18). Such promotion of cholesterol efflux could attenuate deleterious effects of cholesterol overload on macrophage function, as well as limit foam cell formation and enhance cholesterol removal from the vessel wall (4). There is little information, however, as to how efflux enhancement is achieved by endogenous apoE expression in macrophages. For example, can the enhanced efflux produced by the endogenous expression of apoE be reproduced by exogenous addition of apoE to non-expressing macrophages? The answer to this question

Abbreviations: apo, apolipoprotein; ALDL, acetylated low density lipoprotein; BSA, bovine serum albumin; FCS, fetal calf serum; PC, phosphatidylcholine; CD, 2 hydroxy-propyl β cyclodextrin.

[1] To whom correspondence should be addressed.

will provide insight into the mechanism by which the endogenous expression of apoE alters macrophage cholesterol balance. In this series of studies, we have addressed this issue. We do this, first, by establishing the usefulness of the transfected apoE-expressing J774 cell model for these studies, by establishing its similarity to human monocyte-macrophages. We then take advantage of the unique value of such a transfected model to study cholesterol efflux as a function of apoE expression (i.e., in identical macrophages with and without endogenous apoE expression) and in response to addition of exogenous apoE.

MATERIALS AND METHODS

Materials

[^{3}H]cholesterol, [^{14}C]choline chloride, and an enhanced chemiluminescent kit were purchased from Amersham (Arlington Heights, IL). Aprotinin, leupeptin, PMSF, benzamidine, and peroxidase-conjugated rabbit anti-goat IgG were obtained from Sigma (St. Louis, MO). The goat-derived anti-human apoE antiserum was obtained from International Immunology Co. (Murrieta, CA). Egg PC was purchased from Avanti Polar Lipids (Birmingham, AL). Purified apoE from human very low density lipoprotein was obtained from Calbiochem (La Jolla, CA). This apoE had no detectable lipid and was greater than 99% pure by SDS-PAGE. All tissue culture reagents including Dulbecco's modified Eagle's medium, FCS, and neomycin were purchased from Gibco (Grand Island, NY), and all organic solvents including isopropanol, methanol, hexane and chloroform were from Fisher Scientific Co. (Pittsburgh, PA). The BSA used for experiments (Sigma, Cat. # A6003) was >99% pure by SDS-PAGE.

Culture of J774 macrophages and human monocyte-derived macrophages

ApoE-expressing and non-expressing J774 cells have been previously characterized in detail (16, 19–22), and were maintained in DMEM containing 10% FCS and 0.5 mg/ml neomycin at 37°C in an atmosphere of 5% CO_2. The apoE-expressing J774 cells used for these studies secreted 0.5–1.0 μg of apoE per mg cell protein over 24 h depending on the composition of the culture medium. This range is similar to that reported for mature human monocyte-derived macrophages (23). Seven days prior to experiments, the cells were switched to neomycin-free medium. Unless otherwise specified, both apoE-expressing and non-expressing J774 cells were plated and grown in 35-mm dishes at 1.5–2.0 million cells per dish, to 90% confluence. In some experiments, the cells were loaded with cholesterol by incubating with ALDL at 80 μg/ml in DMEM containing 5% FCS for 48 h.

Human monocytes (>94% pure) were isolated by elutriation (24). After differentiation to macrophages for 8 days in 10% pooled human serum and 20% FCS, the cells were placed in DMEM containing 15% FCS with or without ALDL (80 μg protein/ml) and cultured for another 2 days prior to the start of experimental incubations.

Analysis of density distribution for medium lipids and apoE

Confluent cells were washed twice with DMEM plus 0.2% BSA at 37°C, followed by incubation in the same medium for 18 h. At this time, the cells were rinsed again with DMEM alone, and incubated in DMEM containing 0.1% BSA for 24 h. The medium was then collected into tubes containing a protease-inhibitor cocktail (aprotinin, PMSF, leupeptin, and benzamidine), and centrifuged at 2,400 rpm for 15 min, 4°C, to remove any detached cells. The medium was then analyzed by density gradient ultracentrifugation. The density gradient was prepared as follows. Solid KBr was added to the medium to adjust its density to d 1.210 g/ml. Four ml of the medium was then placed in a tube, and the following density solutions was overlaid: 4 ml of d 1.125 g/ml, 3 ml of d 1.063 g/ml, and 2 ml of d 1.006 g/ml. The density gradient was generated by ultracentrifugation in a Beckman SW41 rotor at 35,000 rpm at 4°C for 48 h. After centrifugation, 0.6-ml fractions were collected and the refractory index of each fraction was measured using a Milton Roy refractometer, and the KBr density of each fraction was calculated. The fractions were dialyzed in normal saline containing 1 mm EDTA (pH = 7.4) at 4°C prior to analysis of cholesterol or phospholipid mass, or Western blotting for apoE.

Efflux kinetics assay

J774 cells were labeled with [^{3}H]cholesterol (0.5–2 μCi/ml) and/or [^{14}C]choline (1 μCi/ml) for 24–48 h in DMEM + 5% FCS. [^{3}H]cholesterol was added in ethanol vehicle and the final ethanol concentration in medium was <0.4%. For experiments when cells were preincubated in ALDL to increase cell cholesterol stores, ALDL was included during the labeling incubation. Prior to the start of the efflux incubation, the cells were rinsed three times with DMEM plus 0.2% BSA, incubated at 37°C in DMEM + 0.5% BSA for 2 h, and then rinsed again in DMEM alone. The efflux time-course was initiated by the addition of efflux medium (DMEM + 0.1% BSA) to the culture, and aliquots of medium were taken at the time points indicated in each figure. Medium samples were centrifuged at 2,500 rpm × 15 min at 4°C to pellet detached cells. Lipids were extracted from the supernatant using hexane–isopropranol (16) and quantitated by liquid scintillation counting. Efflux is expressed as a percent calculated as the number of counts present in the medium at each time point divided by the number of cellular counts present at the beginning of the efflux incubation. Cell monolayers were washed three times with ice-cold PBS, dissolved in 0.1 N NaOH, and assayed for protein content using the method of Lowry et al. (25) with BSA as a standard.

Parallel cultures of radiolabeled and unlabeled J774 cells were utilized for simultaneous measurement of cellular cholesterol mass and radioactivity, as well as phospholipid mass and radioactivity. Cholesterol mass was assayed by gas chromatography (16) using coprostanol as internal standard, and phospholipid mass was quantitated according to Bartlett (26).

In some experiments, PC liposomes were used to promote the efflux of cellular cholesterol. These liposomes were prepared as previously described (19) and were filtered through a 0.45-μm filter and stored at 4°C under nitrogen. PC liposomes were added to cells at 1 mg/ml in DMEM with 0.1% BSA.

Immunoblot analysis of apoE

Cell culture supernatants were collected and prepared for immunoblot analysis as previously described in detail (22). For quantitative Western blots, purified exogenous apoE was included to allow generation of a standard concentration curve. ApoE bands were detected using an Amersham enhanced chemiluminescent kit and quantitated by LKB Ultrascan XL densitometry.

RESULTS

Lipid and apoE secretion by apoE-expressing J774 cells and human monocyte macrophages

Table 1 shows the results of a lipid analysis of medium from cultures of apoE-expressing and non-expressing J774

TABLE 1. Lipid analysis of medium from apoE-expressing and non-expressing J774 macrophages

	Medium Lipids	
	Non-expressing Cells	ApoE-expressing Cells
	μg/mg cell protein	
Free cholesterol (FC)	0.33 ± 0.01	0.73 ± 0.06[a]
Phospholipid (PL)	0.42 ± 0.03	0.88 ± 0.10[b]
FC:PL molar ratio	1.52 ± 0.39	1.62 ± 0.18

Confluent J774 cells in T-75 cm^2 flasks were washed three times and incubated in efflux medium. The media were collected after 24 h, and were extracted and assayed as described in the Methods. Results shown are mean ± SD from triplicate samples. Significance values are given comparing non-expressing to apoE-expressing cells.

[a] Difference is significant at $P < 0.001$ level.

[b] Difference is significant at $P < 0.005$ level.

cells. As shown in this table, apoE expression resulted in a more than doubling of the free cholesterol and phospholipid mass secreted from the cells after a 24-h incubation period. Free cholesterol/phospholipid molar ratio in the medium, however, was not altered by apoE expression. An analysis of medium lipid from human monocyte-macrophages is shown in **Table 2**. Here the lipid composition in the medium from apoE-expressing human monocyte-macrophages is compared in the basal or cholesterol-loaded state. In the human monocyte-macrophages, free cholesterol and phospholipid content in the medium, expressed as microgram of lipid per milligram cell protein, was similar to that observed in J774 cells. Free cholesterol/phospholipid molar ratio was also in a similar range. When human monocyte-macrophages were preincubated with ALDL, total cellular cholesterol increased 24% and free cholesterol and phospholipid secretion into the medium increased 92% and 44%, respectively, resulting in an increase of free cholesterol/phospholipid molar ratio. A similar effect of cholesterol loading was observed on the medium content of lipid secreted from apoE-expressing J774 cells and this result is shown in **Table 3**. As shown

TABLE 2. Lipid analysis of medium from human monocyte-macrophages

	Control	Cholesterol-loaded
	cellular lipid (μg/mg cell protein)	
Free cholesterol (FC)	15.78 ± 0.53	18.61 ± 1.92
Cholesteryl ester (CE)	0.59 ± 0.20	1.79 ± 0.75[b]
	medium lipid (μg/mg cell protein)	
Free cholesterol (FC)	0.51 ± 0.01	0.98 ± 0.01[a]
Phospholipid (PL)	0.82 ± 0.24	1.18 ± 0.06[b]
FC:PL molar ratio	1.21 ± 0.37	1.61 ± 0.25

Human monocyte-macrophages were grown in T-75 cm^2 flasks as described in the Methods section. Cells were loaded with cholesterol as described in Methods. Confluent cells were washed three times and incubated in efflux medium. The media were collected after 24 h; media and cells were extracted for lipids and assayed as described in Methods. Results shown are mean ± SD from triplicate samples. Significance values are given comparing control to cholesterol-loaded cells.

[a] Difference is significant at $P < 0.001$ level.

[b] Difference is significant at $P < 0.05$ level.

TABLE 3. Effect of cholesterol loading on medium lipid from apoE-expressing J774 macrophages

	Control	Cholesterol-loaded
	cellular lipid (μg/mg cell protein)	
Free cholesterol (FC)	21.27 ± 0.26	28.24 ± 0.99[a]
Cholesteryl ester (CE)	0.46 ± 0.43	15.36 ± 3.63[a]
	medium lipid (μg/mg cell protein)	
Free cholesterol (FC)	1.17 ± 0.20	2.54 ± 0.12[a]
Phospholipid (PL)	1.07 ± 0.07	1.31 ± 0.09[c]
FC:PL molar ratio	2.11 ± 0.31	3.75 ± 0.25[b]

ApoE-expressing J774 cells were loaded with cholesterol as described in Methods. The cells were then washed three times and incubated in efflux medium. After 24 h, the media were collected, and the lipid from media and cells, was extracted and assayed as described in Methods. Results shown are mean ± SD from triplicate samples. Significance values are give comparing control vs. cholesterol-loaded cells.

[a] Difference is significant at $P < 0.001$ level.

[b] Difference is significant at $P < 0.005$ level.

[c] Difference is significant at $P < 0.05$ level.

here, these cells were studied in a more cholesterol-enriched state; however, changes in medium lipid content were in a direction similar to that observed in human monocyte-macrophages. Cholesterol loading resulted in an increase in free cholesterol and phospholipid secretion from the cells, as well as an increase in free cholesterol/phospholipid molar ratio.

In **Fig. 1** (A-B), we show results of analyses of the density distribution of lipid secreted from apoE-expressing J774 cells. The extra lipid secreted as a result of apoE expression in these cells is organized into a discrete peak with a density range approximating 1.1 to 1.2 g/ml. Figure 1C shows the apoE distribution across this density gradient, and indicates that apoE is found in a similar density range although the shapes of the lipid and apoE peaks are different, suggesting some heterogeneity in the composition of apoE-containing particles secreted from J774 cells. The density distribution of apoE secreted from human monocyte-derived macrophages is also presented in Fig. 1C. The apoE secreted from these cells is found over a density range similar, though not identical, to that observed from apoE-expressing J774 cells; i.e., approximately 1.07–1.22 g/ml.

Kinetics of cholesterol and phospholipid efflux as a function of apoE expression

In **Fig. 2**, the results of a 12-h kinetic study for cholesterol and phospholipid efflux from apoE-expressing and non-expressing J774 cells are presented. Consistent with the mass data shown in Table 1 (which was collected after a 24-h incubation) free cholesterol and phospholipid efflux are both increased by apoE expression. Cholesterol efflux at 12 h was increased from 0.4% to 1.0% (2.5-fold). Also, the time course analysis in this figure shows that differences in efflux for both phospholipid and cholesterol, between apoE-expressing and non-expressing cells, can be detected as early as 3–4 h of study. Further, there is no apparent lag between the first appearance of cholesterol and phospholipid in the medium, suggesting that these two lipid moieties are secreted at the same time with apoE. We

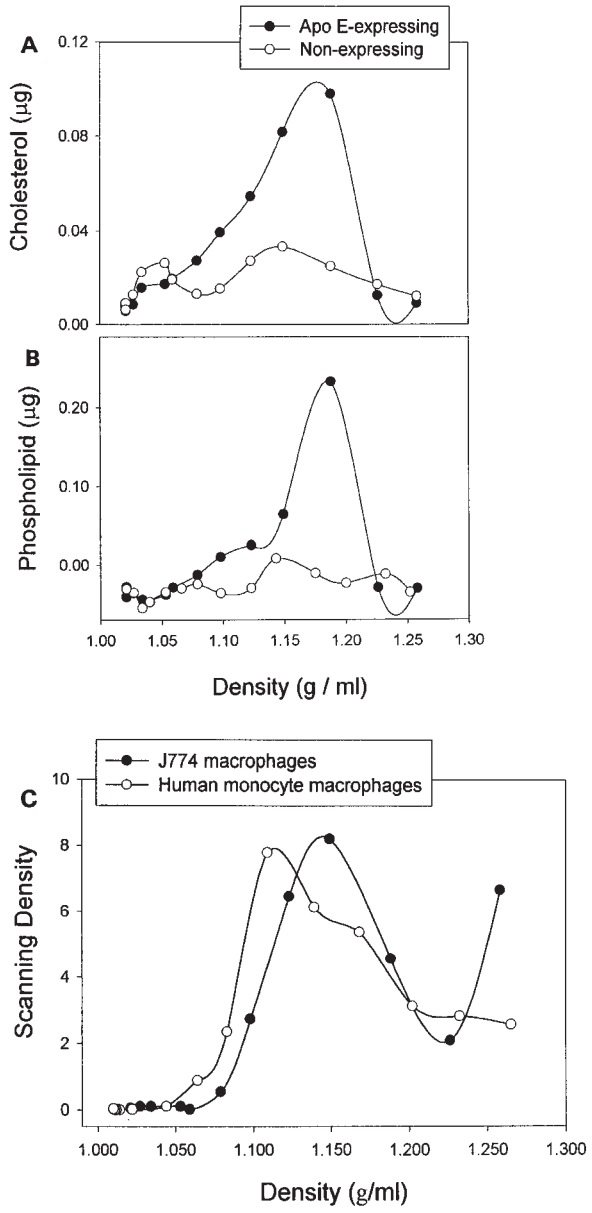

Fig. 1. Density distribution analysis of medium lipids and apoE secreted from macrophages. ApoE-expressing and non-expressing macrophages were incubated in efflux medium for 24 h. The medium collected was subjected to density gradient ultracentrifugation followed by fractionation along the gradient as described in Methods. After dialysis in PBS, lipids were extracted from each fraction and quantified (panel A cholesterol, panel B phospholipid). Density gradient analysis followed by Western blotting of fractions for apoE was also performed using efflux medium collected over a 24-h period from cultured human monocyte-derived macrophages and apoE-expressing J774 macrophages (panel C).

next evaluated the effect of cholesterol loading on efflux kinetics. Loading of cells with cholesterol by preincubation with ALDL widened the difference between apoE-expressing and non-expressing cells so that the increased cholesterol efflux could now be detected as early as 40 min during the efflux period (**Fig. 3**). The result is consistent with the effect of cholesterol loading on the mass data presented in Table 3.

Comparison of the effect of endogenous and exogenous apoE on lipid efflux from macrophages

It has been shown that addition of exogenous apoE can produce efflux from multiple cell types. We next analyzed the effect of addition of exogenous apoE to non-expressing J774 cells. In order to make this evaluation and to make comparisons to the cholesterol efflux observed in apoE expressing cells meaningful, we first determined the concentration of endogenous apoE present in the medium of apoE-expressing J774 cells by quantitative Western blotting. The concentrations of apoE measured in efflux medium from apoE-expressing J774 cells were as follows: at 4 h, 0.133 μg/ml; at 8 h, 0.203 μg/ml; at 12 h, 0.346 μg/ml. We then measured the efflux produced by the addition of exogenous apoE to non-expressing cells at two concentrations that exceeded 0.346 μg/ml: 0.5 μg/ml and 1.0 μg/ml. The results of these studies are shown in **Fig. 4**. Exogenous apoE at 0.5 or 1 μg/ml added at zero time, and for the full 12-h efflux period, resulted in increased cholesterol efflux from non-expressing J774 cells. Cholesterol efflux from these cells appeared to saturate at later time points, even in the presence of 1 μg/ml of exogenous apoE. In spite of this greater than 2-fold increase in the medium concentration of apoE (compared to the concentration found in the medium of apoE-expressing cells) and the fact that cells were cholesterol-enriched, efflux levels saturated at a level below that observed in apoE-expressing cells (Fig. 2). Exogenous apoE added at 0.5 or 1.0 μg/ml to the medium of apoE-expressing J774 cells did not produce additional significant change in cholesterol efflux.

In order to further investigate this apparent difference between the effect of exogenous and endogenous apoE, we performed an experiment in which we collected conditioned medium from apoE-expressing macrophages and added it to non-expressing macrophages in which cholesterol had been labeled. The control for this experiment was the addition of conditioned medium collected from non-expressing cells added to labeled non-expressing cells. Cells were not cholesterol-loaded for this experiment. As shown in **Fig. 5**, the conditioned medium from apoE-expressing cells induced a modest (1.5-fold) increase in efflux which was significant only at the 12-h time point.

It has been proposed that addition of exogenous lipid-free apolipoprotein stimulates cholesterol efflux from cells by enhancing phospholipid efflux (15, 27, 28). We next evaluated and compared the cholesterol efflux produced by exogenous addition, or endogenous expression, of apoE in the presence of a large amount of extracellular phospholipid. The results of experiments with or without exogenous apoE added to non-expressing cells are shown in **Fig. 6**. Phosphatidylcholine (PC) vesicles alone resulted in a large increase of cholesterol efflux (from <1% to 30% at 24 h). The addition of exogenous apoE at 0.5, 1.0, or 2.0 μg/ml with the PC vesicles produced no further increase of cholesterol efflux. This is the result which would be expected if exogenous addition of apoE stimulated cholesterol efflux by a phospholipid-dependent mechanism. We next evaluated the effect of extracellular phospholipid acceptor on the enhanced efflux produced by

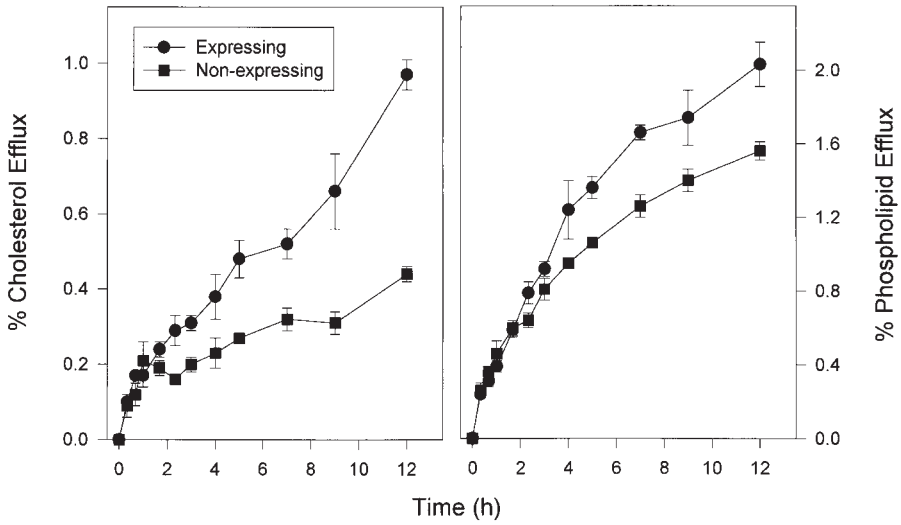

Fig. 2. Kinetic study of cholesterol and phospholipid efflux from apoE-expressing and non-expressing J774 macrophages. ApoE-expressing and non-expressing macrophages were labeled with [^{3}H]cholesterol and [^{14}C]choline as described in Methods. After the equilibration period, efflux medium was added for the start of the efflux incubation (time 0). Aliquots of medium were then taken at each time point indicated and centrifuged to remove floating cells. Lipids were extracted and quantified by liquid scintillation counting as described in Methods. Results shown are the mean ± SD from triplicate samples. Where deviation bars are not visible they are contained within the symbol. For apoE-expressing cells, the total cholesterol mass was 20.6 ± 1.1 μg/mg cell protein, specific activity for cellular cholesterol was 257 ± 13 dpm/ng, and for cellular phospholipid was 17.4 ± 2.7 dpm/ng. For non-expressing cells, the total cholesterol mass was 22.1 ± 0.5 μg/mg cell protein, and the specific activity in cholesterol was 216 ± 9 dpm/ng and the specific activity in phospholipid was 17.7 dpm/ng.

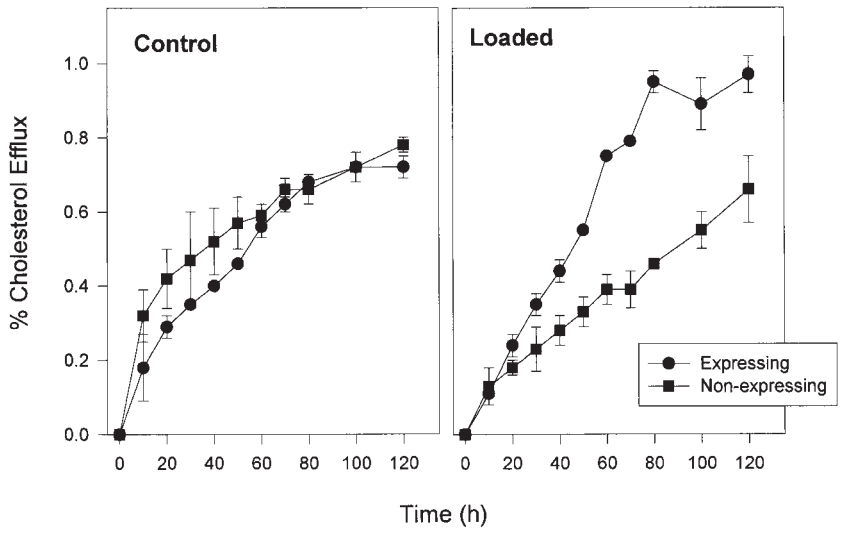

Fig. 3. Two-hour kinetic study of cholesterol efflux from apoE-expressing and non-expressing macrophages after cholesterol loading. J774 macrophages were incubated with or without ALDL (80 μg/ml), along with [^{3}H]cholesterol for 48 h. Efflux was initiated by adding efflux medium to the culture. The medium was sampled at time points indicated and analyzed as described in the legend to Fig. 2. Results shown are mean ± SD from triplicate samples. Where deviation bars are not visible they are contained within the symbol. For apoE-expressing cells without ALDL, the total cholesterol mass was 20.6 ± 3.2 μg/mg cell protein and cholesterol specific activity was 356 ± 3 dpm/ng. For apoE-expressing cells plus ALDL, the total cholesterol was 36.4 ± 0.4 μg/mg cell protein and the specific activity in cholesterol was 81 ± 3 dpm/ng. For non-expressing cells without ALDL, total cholesterol mass was 23.2 ± 5.8 μg/mg cell protein and specific activity was 335 ± 11 dpm/ng. For non-expressing cells plus ALDL, the total cholesterol mass was 40.4 ± 2.2 μg/mg cell protein and the specific activity was 61 ± 4 dpm/ng.

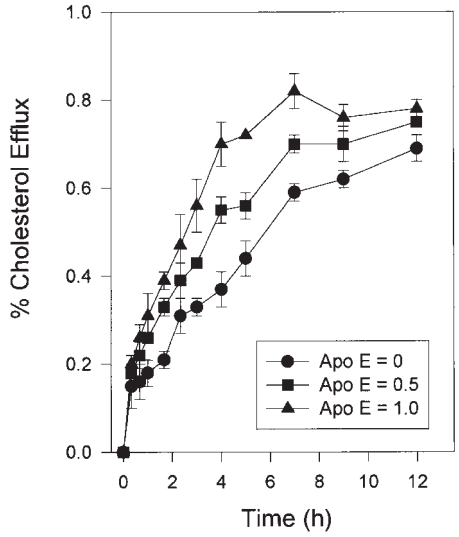

Fig. 4. Effect of exogenous apoE on cholesterol efflux from non-expressing J774 macrophages. J774 cells were cholesterol-loaded by incubation with ALDL (80 μg/ml), along with [^{3}H]cholesterol for 48 h. The efflux incubation was started with various concentrations of purified exogenous apoE as indicated (in μg/ml). Results shown are mean ± SD from triplicate samples. Where deviation bars are not visible they are contained within the symbol. The total cholesterol mass in cells was 50.4 ± 2.2 μg/mg cell protein and specific activity was 11.0 ± 0.6 dpm/ng.

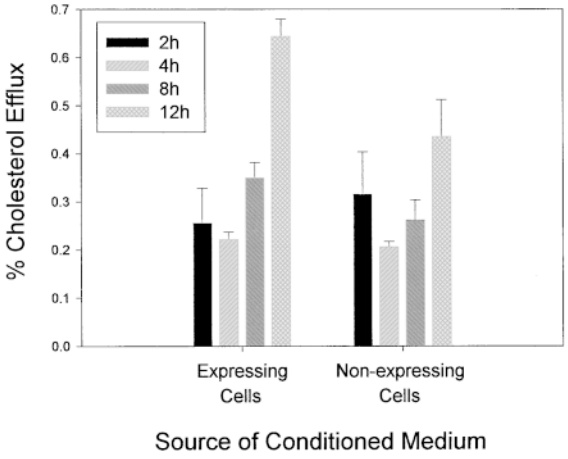

Fig. 5. Effect of conditioned medium from apoE-expressing cells on cholesterol efflux from non-expressing cells. DMEM containing 0.1% BSA was incubated with apoE-expressing J774 cells or non-expressing J774 cells for 12 h. At the end of that time, medium was collected and centrifuged to pellet cellular debris. Supernatants were placed on cultures of non-expressing J774 cells which had been labeled by prior incubation with labeled cholesterol, as described in Methods. Cholesterol efflux was measured as noted in the legend to Fig. 4 at the indicated times. Results shown are the mean ± SD from triplicate samples. The total cholesterol mass was 28.9 ± 2.9 μg/mg, and specific activity was 61.2 ± 9 dpm/ng. Differences between conditioned medium from expressing vs. non-expressing cells were not significant at 2, 4, or 8 h. At 12 h the difference was significant at $P < 0.05$.

endogenous apoE expression by comparing efflux rates in apoE-expressing and non-expressing cells in the presence of PC vesicles. This result is shown in **Fig. 7**. In non-expressing cells, PC vesicles produced a level of efflux at 24 h that was similar to that shown in Fig 6, approximately 30%. In apoE-expressing cells, cholesterol efflux rose to 44% at 24 h in the presence of PC vesicles. The difference between apoE-expressing and non-expressing cells could be detected as early as 90 min.

The results in Fig. 6 indicated that exogenous apoE no longer enhanced efflux in the presence of a large excess of extracellular PC vesicles. In Fig. 7, however, it can be seen that the enhanced efflux produced by endogenous expression of apoE is preserved in the presence of the same concentration of PC vesicles. We next examined whether this observation could be generalized to the cholesterol efflux produced from unloaded cells in the presence of a non-lipid extracellular acceptor for cholesterol. For these experiments we added cyclodextrin (29) to apoE-expressing, or to non-expressing cells, and for the latter cells, included lipid-free apoE, or conditioned medium from apoE-expressing macrophages with cyclodextrin (1.5 mm). In **Fig. 8**, the percent cholesterol efflux at 1, 4, and 8 h is shown for each of these conditions. In the presence of cyclodextrin(CD), non-expressing cells demonstrated cholesterol efflux of approximately 3–4% at all time points. The addition of exogenous apoE (1 μg/ml) with cyclodextrin produced no further increase in efflux at any time point. Cyclodextrin with conditioned medium from apoE-expressing cells produced efflux from non-expressing cells which is modestly higher than without such conditioned medium; most apparent at the 8-h time point (5–6%). In the presence of cyclodextrin, cells that express endogenous apoE demonstrated substantially increased efflux rates compared to non-expressing cells with cyclodextrin at all time points (9–11% vs. 3–4%).

DISCUSSION

In this study, we have taken advantage of a transfected macrophage model in order to study lipid efflux kinetics as a function of endogenous apoE expression. Utilization of this model allowed comparison of lipid efflux rates in non-expressing macrophages to rates in transfected cells that secrete a physiologic amount of apoE. In the first series of studies (Tables 1–3 and Fig. 1), we established the usefulness of this model by demonstrating its similarity to human monocyte-macrophages with respect to free cholesterol and phospholipid secretion, response of lipid secretion to cholesterol loading, and the density of the apoE-containing particle that is secreted. The transfected J774 cell line chosen for this study secretes a physiologic amount of apoE (23). In the initial set of studies reported in apoE-expressing J774 macrophages (16), we measured no increase in cholesterol efflux in the absence of extracellular cholesterol acceptors. However, changing the commercial preparation and extracellular concentration of BSA in the medium allowed us to readily measure the changes in spontaneous cholesterol and phospholipid efflux from these cells as a function of the secretion of phys-

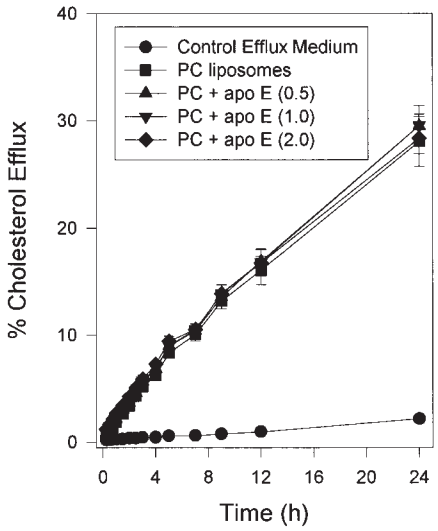

Fig. 6. Effect of PC liposomes on cholesterol efflux produced by exogenous apoE. Cholesterol efflux from cholesterol-loaded non-expressing macrophages was measured in the presence of PC liposomes (1 mg/ml) alone or with various concentrations of exogenously added apoE as shown (in μg/ml). Results shown are mean ± SD from triplicate samples. Where deviation bars are not visible they are contained within the symbol. The total cholesterol mass in cells was 44.0 ± 1.1 μg/mg cell protein and specific activity was 47.1 ± 7.5 dpm/ng.

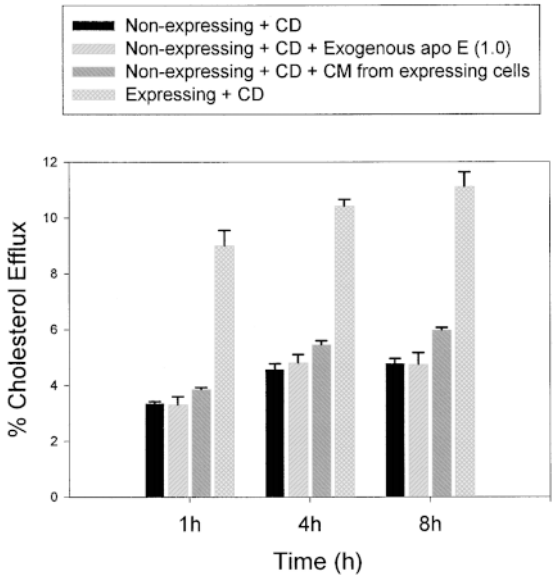

Fig. 8. Comparison of exogenous and endogenous apoE on cyclodextrin-mediated cholesterol efflux. Cholesterol efflux to CD (1.5 mm) was measured from non-expressing macrophages, with or without exogenous apoE or conditioned medium from apoE-expressing macrophages, and from cells expressing endogenous apoE. Values shown are the mean ± SD values for the time points indicated. For non-expressing cells, the cholesterol mass was 25.9 ± 1.4 μg/mg cell protein and the specific activity was 74 ± 4 dpm/ng of cholesterol. For expressing cells, the cholesterol mass was 25.4 ± 1.4 μg/mg cell protein and the specific activity was 69 ± 4 dpm/ng of cholesterol. There were no significant differences produced at any time between non-expressing + CD vs. non-expressing + CD + exogenous apoE. Differences observed for non-expressing + CD + CM, or expressing + CD compared to non-expressing + CD were significant at all time points at $P < 0.005$.

iologic levels of apoE, and to establish their similarity to human monocyte-macrophages in this regard.

In the next series of studies we took advantage of this model to evaluate the effect of apoE expression on the kinetics of cholesterol and phospholipid efflux. While it has been previously demonstrated that endogenous apoE expression enhances cholesterol and phospholipid efflux from macrophages (16–18), kinetic studies have not been reported nor has the effect of cholesterol loading been extensively evaluated. Efflux rates over a 12-h period are shown in Fig. 2. Differences between apoE-expressing and non-expressing cells in lipid efflux can be detected even before 3 h of incubation. In addition, cholesterol and phospholipid appear to be coordinately released into the medium, and the rate of cholesterol efflux as a result of apoE expression appears to accelerate as the incubation is prolonged. In addition, the difference in cholesterol efflux rate between apoE-expressing and non-expressing cells appears to widen as a result of cholesterol loading. The basis for the enhanced difference in lipid efflux rates as a result of apoE expression, when cells are loaded with cholesterol, could be related to alteration in the composition of the cell membranes utilized as a source for lipid efflux (30) or to the increased apoE secretion secondary to the sterol-mediated suppression of intracellular apoE degradation that we have previously demonstrated (22). The results of the isotopic analyses, shown in Figs. 2 and 3, are completely consistent with the mass analyses presented in Tables 1 and 3.

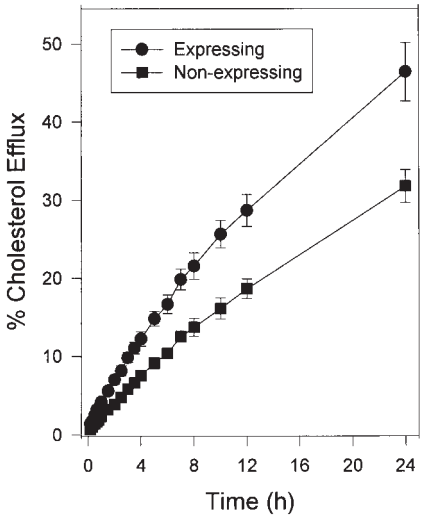

Fig. 7. Effect of PC liposomes on cholesterol efflux produced by endogenous apoE. Cholesterol efflux from cholesterol-loaded apoE-expressing and non-expressing J774 cells was measured in the presence of PC liposomes (1 mg/ml). Results shown are mean ± SD from triplicate samples. Where deviation bars are not visible they are contained within the symbol. The total cholesterol in expressing cells was 47.5 ± 8.3 μg/mg and the specific activity as 47.5 ± 3.2 dpm/ng. The total cholesterol in non-expressing cells was 45.1 ± 1.0 μg/mg and the specific activity was 47.2 ± 3.2 dpm/ng.

As mentioned previously, it has been shown that addition of exogenous apoE to multiple cell types can stimulate cholesterol efflux (12, 13, 31). A potential mechanism which must be considered for our observations, therefore, is that endogenous apoE expression results in medium accumulation of apoE, and thus stimulates efflux by acting as an extracellular acceptor for cholesterol. We believe the results presented in Figs. 4–8, however, support the idea that exogenously added and endogenously expressed apoE enhance cholesterol efflux via divergent mechanisms. The cholesterol efflux produced by addition of exogenous apoE to non-expressing macrophages, even when added at a level which far exceeds the time-averaged concentration produced by endogenous apoE expression, does not reach the level of cholesterol efflux produced by the endogenous expression of apoE in this cell model. In fact, cholesterol efflux to exogenous apoE appears to saturate at this lower level of efflux, in spite of the much higher medium concentration of exogenously added apoE. Further, the addition of PC vesicles or CD completely eliminates the enhanced efflux due to exogenous apoE. However, the cholesterol efflux produced by endogenous apoE expression remains intact and is even enhanced (in absolute incremental change) in the presence of these potent extracellular cholesterol acceptors.

We believe there are several unique mechanisms which can be considered for the effect of endogenous apoE expression on macrophage cholesterol efflux. One potential mechanism could relate to a more efficient production of a cell-surface pool of apoE by endogenous expression. Endogenously synthesized apoE may be more efficiently sequestered at the cell surface due to an increased exposure to low affinity binding sites at the cell surface during passage through the pericellular space, or due to a unique apoE conformation related to differences in glycosylation or lipidation between endogenous and exogenous apoE. A high concentration of apoE at the cell surface could, then, facilitate lipid desorption from the plasma membrane. This mechanism could explain why addition of exogenous apoE, at levels similar to those produced by endogenous expression, fails to stimulate additional efflux from apoE-expressing cells, because endogenous and exogenous apoE would presumably compete for the same cell surface binding sites. This mechanism would also be consistent with previous reports indicating that addition of apoE antisera reduces, but does not eliminate, cholesterol efflux from human monocyte-macrophages (17). The residual efflux from these cells in the presence of apoE antisera could be related to a second potential unique mechanism, an intracellular role for endogenous apoE-mediated efflux. A potential intracellular role for the enhanced lipid efflux due to endogenous synthesis of apoE is consistent with observations that a substantial amount of apoE is never secreted from macrophages, and that the intracellular processing and degradation of apoE is modulated by the macrophage intracellular lipid milieu (22). It also is consistent with previous observations that maximal cholesterol efflux from cells depends on the transport of cholesterol from intracellular compartments to the plasma membrane (32–34). Also in line with this mechanism are recent observations that suggest that apoE may have an intracellular function in hepatocytes (35). In recently reported studies, endogenous apoE expression in hepatocytes was uniquely required for remnant lipoprotein clearance in the absence of the LDL receptor. A third mechanism which can be considered is the extracellular accumulation of unique apoE/lipid particles when apoE is endogenously synthesized and secreted, versus its exogenous addition. The results in Fig. 8, showing more efflux from non-expressing cells in the presence of CD + conditioned medium from expressing cells compared to CD + exogenous lipid-free apoE, would support this mechanism. However, the small magnitude of the difference produced by the conditioned medium in Fig. 8, and in Fig. 5, argues against differences in extracellular particle composition being the major determinant for our observations. It is still possible, however, that the collection of conditioned medium precludes detecting an effect of a nascent macrophage-derived apoE particle which potently enhances cholesterol efflux. Therefore, though less likely, this potential mechanism is not completely excluded by our results. The final explanation to consider is that endogenous apoE produces efflux from a different subcellular cholesterol pool, compared to the addition of exogenous apoE. While up to 90% of cellular cholesterol is found in the plasma membrane, there may be poorly characterized intracellular pools (36, 37). In our studies, we assumed uniform labeling of all cellular cholesterol and made no attempt to quantify differences in mass or specific activity of subcellular pools. It is unlikely that apoE expression would produce differences in labeling of subcellular pools based on the close agreement of our isotope analyses and mass analyses (Tables 1 and 3 compared to Figs. 2 and 3). However, it remains possible that intracellular apoE resulting from endogenous expression uses a more highly labeled cellular pool as a substrate for efflux than extracellular apoE. While our data do not completely rule out this possibility, the experiments in which there are high levels of efflux (in the presence of extracellular acceptors such as phosphatidylcholine liposomes or cyclodextrin) make it unlikely that the observed differences in cholesterol efflux between exogenous and endogenous apoE are due to differences in the utilization of a minor pool as a substrate for efflux. Based on our data, we currently favor an intracellular or cell-surface mode of action for the effect of endogenous apoE expression on lipid efflux.

In summary, in this study we have further validated the usefulness of the transfected J774 macrophage model for the study of apoE-induced cholesterol efflux, and analyzed the effect of cholesterol loading on apoE-induced lipid efflux. We have presented studies analyzing the effect of apoE expression and exogenous apoE addition on cholesterol efflux kinetics from macrophages. These studies allowed the detection of differences between the cholesterol efflux produced by endogenous expression versus exogenous addition of apoE in terms of level of efflux achieved. While the relative efficacy of exogenously added

apoproteins compared to endogenously expressed apoproteins in producing cholesterol efflux from macrophages may vary depending on the model and in vivo vessel wall micro-environment, the difference in efficacy we demonstrated in the J774 model provides evidence for production of efflux by divergent mechanisms. The saturability of efflux and the response to extracellular cholesterol acceptors was also different between endogenous expression and exogenous addition of apoE. These differences provide evidence that endogenous apoE expression by macrophages enhances cholesterol efflux by mechanisms distinct from those utilized by exogenous addition of apoE.

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