A Two-Step Mechanism for Free Cholesterol and Phospholipid Efflux from Human Vascular Cells to Apolipoprotein A-1†

Phoebe E. Fielding,*,‡,§ Koji Nagao,§ Hideki Hakamata,§ Giovanna Chimini,|| and Christopher J. Fielding§,⊥

Cardiovascular Research Institute and Departments of Medicine and Physiology, University of California, San Francisco, California 94143, and Centre d'immunologie, INSERM-CNRS de Marseille Luminy, 13288 Marseille, France

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ABSTRACT: Smooth muscle and endothelial cells in vivo are quiescent yet exposed to high levels of lipoprotein lipids. Phospholipid (PL) and free cholesterol (FC) efflux maintain homeostasis. Smooth muscle cells (SMC) expressed high levels of ABC-1 transporter mRNA, and glyburide-dependent PL and FC efflux to apolipoprotein A-1 (apo A-1), the major protein of high-density lipoprotein. FC efflux was inhibited by vanadate and okadaic acid, while PL efflux was not. Phosphatidylcholine was the major PL transferred by both cell types. Stimulation of phosphatidylserine efflux, redistributed within the membrane by this transporter, was only minimally increased. Umbilical vein and aortic endothelial cells expressed little ABC-1 mRNA, nor did these cells promote either PL or FC efflux in response to the presence of apo A-1. To investigate the mechanism of ABC-1-dependent lipid efflux from these cells, apo A-1 was preincubated in the presence of unlabeled SMC or fibroblasts, and the conditioned medium was then transferred to endothelial cells. This medium catalyzed the efflux of FC but not of PL from endothelial cells. Such FC efflux was resistant to glyburide but inhibited by okadaic acid and vanadate. The data suggest that ABC-1-dependent PL efflux precedes FC efflux to apo A-1 and that the complex of apo A-1 and PL is a much better acceptor of FC than apo A-1 itself. Inhibition of FC but not PL efflux by vanadate and okadaic acid suggests these transfers involve different mechanisms.

The FC[1] content of cells cultured in normal (lipoprotein-containing) media, when cellular LDL receptors are down-regulated, appears to be mainly determined by a balance between the uptake of low-density lipoprotein (LDL) FC and the efflux of FC to high-density lipoprotein (HDL) (*1*). A minor, lipid-poor fraction of HDL, with prebeta-electrophoretic mobility (prebeta-HDL) and containing as protein only apolipoprotein A-1 (apo A-1), has been identified as a major initial acceptor of cell-derived FC (*2*, *3*) and phospholipid (PL) (*4*). Prebeta-HDLs are enriched in the subendothelial space, particularly in the area of atherosclerotic lesions (*5*), suggesting an active role for the FC efflux pathway in the vascular bed. Efflux of FC to HDL was responsive to cell FC levels (*6*). This response was linked to caveolae, microdomains rich in FC and sphingolipids at the surface of most cells, and enriched on endothelial cells (*7*). Caveolins are major structural proteins of caveolae, and the expression of a major caveolin (caveolin-1) was positively correlated with FC efflux (*8*, *9*). A homeostatic mechanism was recently proposed such that if cell FC levels are increased, for example, via increased extracellular levels of LDL FC, preformed caveolin moves from intracellular pools to the cell surface, caveolin synthesis is upregulated transcriptionally, and caveolae, caveolar FC, and FC efflux all increase as the level of cell FC is normalized (*10*). Consistent with this, FC efflux and caveolin expression were both reduced following the S phase in synchronized human skin fibroblasts (*11*). In cells with few caveolae, such as lymphocytes and many human cancer cell lines, the same function may be played by cell surface lipid "rafts" with a similar lipid composition (*12*). However, many features of the transfer of FC and PL from the cell surface to lipid-poor apo A-1 lipoprotein particles remain unclear. These include whether transfer of FC and PL takes place simultaneously, if the same or different pathways are utilized, and if efflux involves the simultaneous transfer of preformed complexes of plasma membrane lipids ("microsolubilization") or the sequential transfer of individual lipid molecules from different microdomains of the cell surface to the same acceptor (*13*, *14*).

The plasma membrane contains a number of ABC family lipid transporters (*15*). One of these (ABC-1) was recently shown to be defective in human Tangier disease (*16*−*19*). Plasma HDL cholesterol and PL were both almost completely absent in this inherited disorder (*20*). On the basis of this, it has been concluded that ABC-1 transported both PL and FC across the plasma membrane. ABC-1 has also been previ-

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‡ Department of Medicine, University of California.
§ Cardiovascular Research Institute, California.
|| INSERM-CNRS de Marseille Luminy.
⊥ Department of Physiology, University of California.

[1] Abbreviations: PL, phospholipid; FC, free cholesterol; ABC, ATP-binding cassette; SMC, smooth muscle cells; HAEC, human aortic endothelial cells; HUVEC, human umbilical vein endothelial cells; HDL, high-density lipoprotein; LDL, low-density lipoprotein.

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ously reported to be active in the secretion of iodide and interleukin-1β and in the phagocytosis of apoptotic cell ghosts by macrophages (*21*−*23*). Tangier cells are defective in PL and FC efflux (*24*), but a direct role for ABC-1 in the secretion of either lipid has not been demonstrated. The recent report of selective inhibitors of ABC-1 transporter activity (*19*, *21*) offered a novel approach to determining whether the PL and FC efflux mechanisms were the same.

Because of the significance of FC efflux in regulating cell surface properties, particularly in vascular cells (*25*), the formation of HDL particles from apo A-1 by endothelial and vascular smooth muscle cells was investigated. The results suggest that a two-step mechanism involving sequential contributions of the ABC-1 transporter and caveolae could mediate much of HDL formation from these cell lines.

EXPERIMENTAL PROCEDURES

Materials. Apo A-1, glyburide (glibenclamide), and okadaic acid were purchased from CalBiochem. Bovine serum albumin (BSA), egg phosphatidylcholine, and FC were from Sigma (St. Louis, MO). Unlabeled FC and egg phosphatidylcholine were from the same source. Human aortic endothelial cells (HAEC), human umbilical vein endothelial cells (HUVEC), and human aortic smooth muscle cells (SMC) were from Clonetics (San Diego, CA). SMC were from a 2-year-old male donor; HUVEC were derived from male and female newborns, and HAEC were from female donors (50−55 years of age). No differences were identified in the lipid efflux properties of HUVEC and HAEC in the studies identified below. Erythrocytes were obtained from the blood of fasting normal donors. [1,2-^{3}H]Cholesterol, [^{3}H]-choline, [^{32}P]phosphate, ^{125}I-labeled sodium iodide, and [^{32}P]-dCTP were all from NEN (Boston, MA). High-molecular weight dextran (dextran T-500) was from Pharmacia (Parsippany, NY). Polyorthovanadate was prepared from sodium vanadate (*26*).

Cell Culture. HAEC, HUVEC, and SMC were grown in EBM (Clonetics) supplemented with fetal bovine serum (FBS). Normal human skin fibroblasts were grown in DMEM and 10% FBS. For individual experiments, cells were plated in 3.5 cm Corning dishes, and grown to near (>90%) confluence.

In some experiments, cells were labeled with [^{3}H]FC (2−5 μCi per dish) for 24 h. They were then incubated for an additional 24 h in DMEM or EBM with 2 mg/mL BSA. To determine the FC specific activity, basal (unloaded) or FC-loaded washed cells were solubilized with 0.1 N NaOH and extracted with chloroform and methanol. Portions of the chloroform phase were collected for liquid scintillation spectrometry, and for enzymatic determination of FC mass (*27*). In some cases, the cells had been FC-loaded (24 h) in EBM containing 2 mg/mL albumin and 30 μg/mL FC (*28*).

In other studies, cells were labeled for 24 h in phosphate-free medium together with 2−10 μCi of [^{32}P]phosphate or with 2 μCi of [^{3}H]choline. Dishes were then transferred to BSA (2 mg/mL) in the same medium for 24 h. To determine the [^{32}P]PL specific activity, washed cells were solubilized with NaOH and extracted with chloroform and methanol, in the presence of 10 mM $CaCl_2$ to facilitate partition of PL into the chloroform phase. Portions of the chloroform phase were collected for liquid scintillation counting and to determine lipid phosphorus mass (*29*). The specific activity was calculated assuming a mean PL molecular weight of 750. More prolonged (48 h) equilibration with either the ^{3}H or ^{32}P label was without effect on subsequent lipid specific activities, indicating that equilibration had been reached under the conditions described here.

Whole blood was labeled with [^{3}H]FC as previously described (*4*). The erythrocyte fraction was purified by centrifugation (three times at 2000*g* for 30 min) and finally resuspended in a phosphate-buffered saline solution (pH 7.4).

PL Efflux. Efflux was assayed as the rate of transfer of the [^{32}P]- or [choline-^{3}H]PL label from cells into culture medium containing 2 mg/mL BSA in the presence of lipid-free apo A-1 (*24*). Assays were carried out in the presence of 10 μg/mL apo A-1 except where indicated. Under these conditions, the efflux of PL was linear for at least 5 h at 37 °C. After incubation, the medium was centrifuged to remove any dissociated cells and then extracted with chloroform and methanol as described above. Portions of the chloroform phase were assayed by liquid scintillation spectrometry to quantitate the PL label. Efflux into EBM-BSA without apo A-1 was subtracted from values obtained with complete assay medium. In some experiments, BSA was replaced with high-molecular weight dextran (0.25 mg/mL) without any effect on the rate of apo A-1-dependent efflux.

FC Efflux. Efflux from [^{3}H]FC-labeled cells was assessed under the conditions described above for PL, except that the solvent extraction step was omitted. To compare efflux rates of both lipid classes between experiments, efflux rates were in each case calculated as the number of picomoles of FC or PL transferred per hour per microgram of cell FC. In some experiments with endothelial cells, phosphatidylcholine or mixtures of phosphatidylcholine and FC dried under N_2 from an ethanolic solution were dispersed by sonication (*30*) prior to inclusion in EBM, in the absence of apo A-1, at a concentration indicated in individual experiments. In other experiments, dishes of unlabeled SMC were incubated (5 h at 37 °C) with apo A-1 (10 μg/mL) ("preconditioned medium") in the presence or absence of inhibitor as indicated for individual experiments. This medium was then transferred to endothelial cells prelabeled with [^{3}H]FC, and its effects on FC efflux were determined.

Thin-Layer Chromatography. To characterize the contributions of different phospholipids to total efflux, chloroform extracts of labeled PL were fractionated by thin-layer chromatography (TLC) on Whatman LK6 silica gel plates developed in a chloroform/methanol/2-propanol/0.25% aqueous KCl/triethylamine mixture (30/9/25/6/18, v/v) (*31*). ^{32}P-labeled lipids were visualized by autoradiography using Kodak X-OmatAR film and quantitiated with a computerized scanning densitometer (ImageQuant, Molecular Dynamics, Sunnyvale, CA). Individual lipid classes were identified from their comigration with pure standards of major cellular and plasma phospholipids.

The recovery of each PL class through the extraction procedure was determined by fractionating total PL from ^{32}P-labeled cells by TLC as described above. Each was recovered from the gel with a chloroform/methanol/water mixture (65/35/5, v/v), and the phases were separated in the presence of 10 mM $CaCl_2$ as described above. On average, 98.9% of the total PL label was recovered in the chloroform phase. Recovery of individual phospholipid classes varied from

Table 1: PL Efflux from Human Fibroblasts and Vascular Cells[a]

	PL efflux [ng h^{-1} (μg of cell FC)$^{-1}$]		
cell type	apo A-1 only	apo A-1 and glyburide	apo A-1 and vanadate
fibroblasts	37.2 ± 6.9 (1.0)	0.6 ± 0.3 (0.02)	34.2 ± 5.0 (0.9)
SMC	92.4 ± 6.6 (1.0)	10.6 ± 8.8 (0.1)	65.5 ± 9.8 (0.7)
HAEC	0.2 ± 0.1	0.0	0.1 ± 0.1

[a] Values are means ± one standard deviation of triplicate determinations. Values in parentheses are rates relative to apo A-1 only. Rates that are shown are for unloaded cells, labeled and assayed as described in Experimental Procedures. The apo A-1 concentration was 10 μg/mL in all experiments. Glyburide (200 μM) or vanadate (1 mM) was included as indicated. Assays were linear over the course of 5 h at 37 °C. Values in parentheses represent the proportion of original activity retained in the presence of the inhibitor shown.

96.3% (phosphatidylethanolamine) to 100% (phosphatidylserine and sphingomyelin).

Northern Blotting. Total RNA was extracted from cell dishes using RNeasy kits (Qiagen, Chatsworth, CA). Five micrograms of total RNA was applied to 1% agarose−formaldehyde gels. Following electrophoresis, it was transferred to 2 μm pore nylon screens by capillary blotting. To detect and quantitate ABC-1 mRNA, full-length mouse ABC-1 transporter cDNA (*23*) was subcloned into pcDNA3 (In Vitrogen, Carlsbad, CA). ^{32}P-labeled random primed cDNA was hydribized with the blots, and ABC-1 mRNA was quantitated densitometrically after autoradiography.

RESULTS

PL Efflux from Fibroblasts and SMC. PL efflux was assayed from [^{32}P]- or [^{3}H]choline-labeled cells equilibrated as described in Experimental Procedures (Table 1). Efflux was linear over the 5 h assay. Under equivalent conditions, PL efflux was approximately 2.5-fold more rapid from SMC than from fibroblasts.

The stimulation of PL efflux observed in the presence of apo A-1 was strongly inhibited by glyburide (200 μM), an inhibitor of ABC-1-mediated transport (*21*). The extent of inhibition by glyburide was similar with fibroblasts and SMC (90 ± 7 vs 98 ± 3%, Table 1). In contrast, glyburide was without significant effect (<5% inhibition, three experiments) on the basal rate of PL efflux which occurred in the absence of apo A-1. Vanadate had a much smaller effect on PL efflux than glyburide (Figure 1), consistent with previous reports (*21*). Okadaic acid (OKA), an inhibitor of FC efflux from fibroblasts (*32*), was without significant effect (<5% inhibition, three experiments) on PL efflux from either SMC or fibroblasts, also in agreement with previous reports (*21*). When the level of stimulation of PL efflux was determined as a function of medium apo A-1 concentration, the apparent K_m was 0.3 ± 0.1 μg/mL (10.5 ± 3.0 nM, three experiments).

The extent of apo A-1-dependent stimulation on the efflux of individual PL species from fibroblasts and SMC, and its level of inhibition by glyburide, was determined following TLC (Figure 2, left and center). In the absence of apo A-1, much of the label released to the culture medium comigrated with lysophosphatidylcholine. In contrast, in the presence of apo A-1, phosphatidylcholine was the major PL whose efflux was stimulated. In the case of fibroblasts, the efflux of phosphatidylinositol was also enhanced, although to a lesser extent. By contrast, the level of phosphatidylinositol in medium from SMC incubated with apoA-1 was much lower (Figure 2, center).

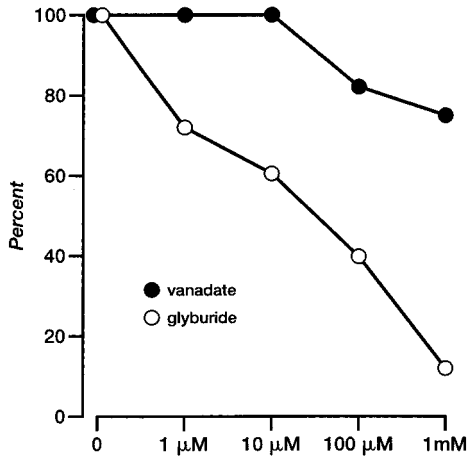

FIGURE 1: Concentration dependence of inhibition of phospholipid efflux by glyburide (○) and vanadate (●). Incubations were carried out with SMC for 5 h at 37 °C as described in Experimental Procedures in the presence of apo A-1 (10 μg/mL) and inhibitor as indicated. Values are means of duplicate assays.

The effect of apo A-1 on the efflux of each PL fraction was determined by comparing its recovery after incubation in the presence or absence of apo A-1. The data were expressed in percentage terms. The effect of glyburide on the efflux of each PL fraction was determined from the recovery of that PL after incubation with apo A-1 in the presence or absence of glyburide (200 μM) and expressed in the same way. The specificities among PL fractions of the effects of apo A-1 activation and glyburide inhibition were then compared (Figure 3).

In the case of both fibroblasts and SMC, glyburide quantitatively reversed the effect of apo A-1 in terms of not only the total PL efflux but also the pattern of PL species released. Seventy percent of the additional PL label released from SMC in the presence of apo A-1 was phosphatidylcholine. The remaining label was recovered in sphingomyelin and lysophosphatidylcholine. When glyburide was added together with apo A-1, the changes made by apo A-1 alone were almost quantitatively reversed. For fibroblasts, phosphatidylcholine represented about 50% of the additional PL label released in the presence of apo A-1. This increase was also reversed when glyburide was present along with apo A-1 (Figure 3). These data indicate that glyburide reverses the effect of apo A-1 in terms of individual PL classes released into the medium as well as total PL.

PL Efflux from HAEC and HUVEC. In contrast to the effects seen with fibroblasts and SMC, apo A-1 did not stimulate additional PL efflux from HAEC (Table 1). Comparable data were obtained with HUVEC. The PL released into the medium, like that of SMC and fibroblasts in the absence of apo A-1, was mainly lysophosphatidylcholine. Inclusion of apo A-1 in the extracellular medium was without effect on the amount of released PL. Glyburide was without effect on the efflux of ^{32}P-labeled PL from endothelial cells (Figure 2, right).

FC Efflux from Fibroblasts and SMC. Apo A-1 promoted efflux of both PL and FC from SMC and fibroblasts. The rate of FC efflux was about 15-fold lower than that of PL in fibroblasts and about 7-fold lower in SMC. The rate of FC

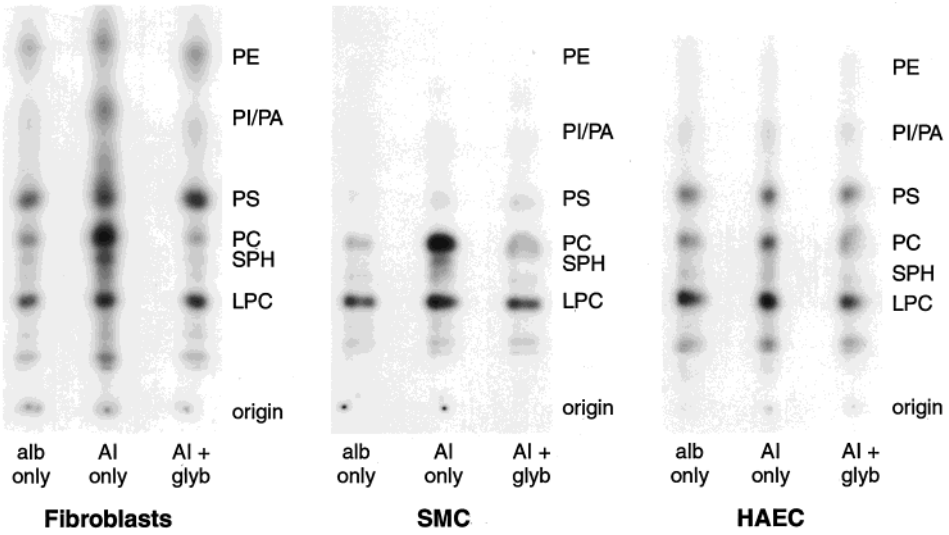

FIGURE 2: (Left) Phospholipids transferred from human skin fibroblasts to extracellular medium in the presence or absence of apo A-1. ^{32}P-labeled cells were incubated in the presence of BSA medium (without FBS) for 5 h at 37 °C. The medium was extracted with chloroform and methanol. Portions of the chloroform phase were fractionated by TLC as described in Experimental Procedures, together with lipid standards. Component labeled phospholipids were visualized by autoradiography and then quantitated by scanning densitometry. From left to right: BSA medium only (no apo A-1), medium and apo A-1 (10 μg/mL) only, and medium, apo A-1, and glyburide (200 μM). (Center) Phospholipids transferred from SMC in the presence or absence of apo A-1. The cells were ^{32}P-labeled, incubated, extracted, and chromatographed as described for the left panel. From left to right: BSA medium only (no apo A-1), medium and apo A-1 (10 μg/mL), and medium, apo A-1, and glyburide (200 μM). (Right) Phospholipids transferred from HAEC in the presence or absence of apo A-1. The cells were ^{32}P-labeled, incubated, extracted, and chromatographed as described for the left panel. From left to right: BSA medium only (no apo A-1), medium and apo A-1 (10 μg/mL), and medium, apo A-1, and glyburide (200 μM).

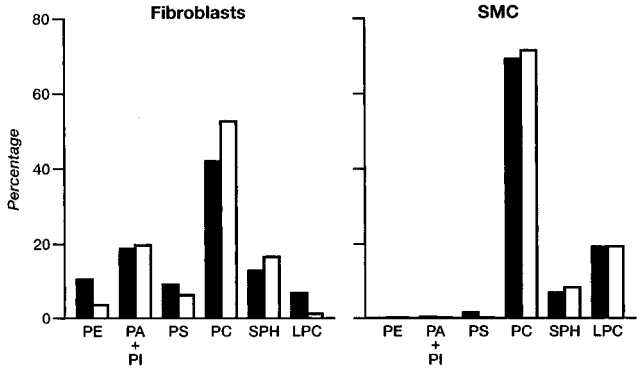

FIGURE 3: Changes in the proportions of different [^{32}P]PLs transferred to the medium from fibroblasts (left) or SMC (right): black bars, (BSA medium + apo A-1) minus (BSA medium − apo A-1); and white bars, (BSA medium + apo A-1) minus [BSA medium + apo A-1 + glyburide (200 μM)]. After incubation for 5 h at 37 °C, the medium was extracted with chloroform and methanol. TLC were carried out on portions of the chloroform phase as described in Experimental Procedures. After autoradiography, the level of each lipid present was expressed in densitometric units. Changes in the lipid pattern are expressed as a percentage of the total change observed between each pair of experimental conditions.

efflux was about 5-fold lower in fibroblasts than in SMC in these experiments (Table 2). [^{3}H]FC efflux, like that of PL, was strongly (80−90%) inhibited by glyburide. In contrast to its smaller effect on PL efflux, 1 mM vanadate strongly (~80%) inhibited FC efflux from both SMC and fibroblasts. When the rate of FC efflux was measured as a function of medium apo A-1 concentration, the apparent K_m was 1.5 ± 0.2 μg/mL (53 ± 7 nM).

In previous studies, FC efflux was significantly inhibited by okadaic acid, an inhibitor of protein phosphatases that downregulates caveolin expression (*32*). Unlike PL efflux, which was unaffected (<3% inhibition, three experiments),

Table 2: FC Efflux from Human Fibroblasts and Vascular Cells[a]

	FC efflux [ng h^{-1} (μg of cell FC)$^{-1}$]		
cell type	apo A-1 only	apo A-1 and glyburide	apo A-1 and vanadate
fibroblasts	2.4 ± 0.5 (1.0)	0.5 ± 0.2 (0.2)	0.5 ± 0.2 (0.2)
SMC	12.8 ± 0.6 (1.0)	1.3 ± 0.3 (0.1)	2.9 ± 0.1 (0.2)
HAEC	0.1 ± 0.0	0.0	0.0

[a] Values shown are means ± one standard deviation of triplicate determinations. Values in parentheses are rates relative to apo A-1 only. Assay conditions are as described in the footnote of Table 1. Values in parentheses represent the proportion of original activity retained in the presence of the inhibitor shown.

FC efflux from both SMC and fibroblasts to apo A-1 was strongly inhibited (95 ± 2%) in the presence of 1 μM okadaic acid (n = 3).

FC Efflux from HAEC and HUVEC. Apo A-1 did not significantly stimulate the efflux of FC from endothelial cells, in contrast to its effect with fibroblasts and SMC, nor was there any effect of vanadate (Table 2). These data indicated that SMC and fibroblasts promoted apo A-1-dependent efflux of both PL and FC, while HAEC and HUVEC lacked detectable levels of either activity.

FC Efflux from Erythrocytes. FC efflux from these cells is considered to occur by diffusion without involvement of cell surface lipid-binding proteins (*33*). The transfer of [^{3}H]-FC from erythrocytes to DMEM (pH 7.4) containing 2 mg/mL serum albumin was assessed in the presence and absence of apo A-1 (10 μg/mL). No effect of apo A-1 was observed, nor did the inclusion of glyburide (200 μM) or vanadate (1 mM) modify the rate of efflux observed in the presence of DME and albumin medium.

These data suggest that the mechanism of FC efflux from erythrocytes differs significantly from that seen in fibroblasts, SMC, and endothelial cells. The same conclusion was

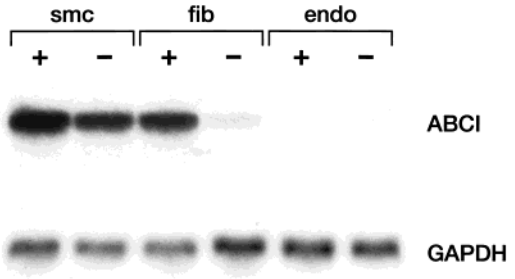

FIGURE 4: Northern blots of mRNA from fibroblasts, SMC, and HAEC. Cells had been either grown in normal medium as described in Experimental Procedures (−) or preloaded (24 h) with 2 mg/mL BSA complexed with 30 μg/mL FC (+). FC loading increased the cell FC content by 1.9-fold (fibroblasts), 2.7-fold (SMC), and 2.3-fold (HAEC). Five micrograms of total RNA was applied to each lane. After electrophoretic fractionation, hybridization was carried out with ^{32}P-labeled full-length murine ABC-1 cDNA. The labeled complexes were visualized by autoradiography and quantitated by densitometry. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA as an internal standard was visualized with a GAPDH probe under the same conditions (*11*). In overexposed gels, a faint endothelial cell signal was visible but not upregulated by FC.

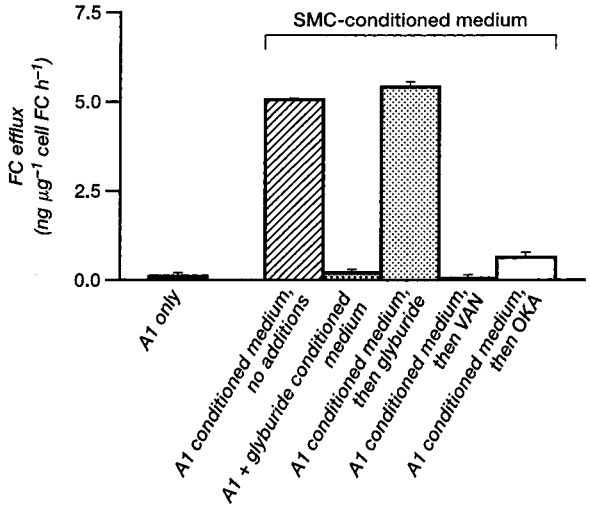

FIGURE 5: Incubation of [^{3}H]FC-labeled HAEC with preconditioned medium from unlabeled SMC. Dishes of SMC were incubated in BSA medium with apo A-1 for 5 h at 37 °C. The SMC-conditioned medium was then transferred to washed HAEC. After incubation for an additional 5 h, the HAEC medium was assayed for FC radioactivity. Some dishes of labeled HAEC were incubated directly with apo A-1 in BSA medium. Glyburide (200 μM) was added either to the unlabeled SMC or after preincubation directly to the HAEC. Vanadate (1 mM) or okadaic acid (1 μM) was added directly to the HAEC. A representative experiment (of five) is shown. As described in the Results, conditioned medium prepared from SMC in the presence of vanadate, and then dialyzed overnight, did not inhibit FC efflux from HAEC.

reached previously with respect to FC efflux to native plasma (*4*).

ABC-1 mRNA Levels. ABC-1 mRNA was easily detected by Northern blotting in SMC extracts. The level of expression of ABC-1 in fibroblasts was 10-fold lower, but still detectable (Figure 4). In contrast, under the same conditions, ABC-1 could not be detected using the same mass of endothelial cell total RNA.

ABC-1 mRNA expression is strongly upregulated by cholesterol loading in both macrophages (*34*) and immortalized fibroblasts (*19*). When the FC content of fibroblasts was raised 2-fold by preincubation with the FC−albumin complex, ABC-1 mRNA levels were upregulated 10−12-fold. Under the same conditions, there was a smaller (2-fold) increase in the level of expression of this message in SMC, so the level in FC-loaded fibroblasts was about one-half of that in FC-loaded SMC. No detectable ABC-1 message was observed in RNA from FC-loaded HAEC and HUVEC.

PL and FC Transport into Conditioned Medium. The data above indicate that both endothelial cell lines substantially lack ABC-1 mRNA as well as ABC-1-dependent PL and FC efflux activities, despite earlier observations that FC efflux from human endothelial cells into native plasma was both rapid and apo A-1-dependent (*35*). This might indicate that the ABC-1 transporter promoted the efflux of both FC and PL from fibroblasts and SMC, while FC efflux from endothelial cells was via a distinct, ABC-1-independent pathway. Alternatively, FC efflux from endothelial cells might represent the second of two steps in which apo A-1 first bound to PL derived from ABC-1 transporter activity from other cells, such as SMC or fibroblasts.

To distinguish these possibilities, dishes of unlabeled SMC (containing 10 ± 1 μg of cell FC) were incubated with apo A-1 in the presence or absence of glyburide. The conditioned medium was then transferred to [^{32}P]PL- or [^{3}H]FC-labeled HAEC or HUVEC monolayers, in the presence or absence of glyburide, okadaic acid, or vanadate.

Apo A-1 in SMC-conditioned medium, like apo A-1 added directly to endothelial cells labeled with ^{32}P, did not promote additional efflux of PL (<3% stimulation of efflux in the absence of apo A-1, four experiments). There was no effect of glyburide, vanadate, or okadaic acid when these were included with the conditioned medium. In contrast, when apo A-1 medium preconditioned by incubation with either fibroblasts or SMC was added to [^{3}H]FC-labeled HAEC, a significant stimulation of the rate of FC efflux was observed (Figure 5). The same result was obtained with HUVEC (data not shown). The rates of FC efflux that were obtained were about one-half of the rates observed with [^{3}H]FC-labeled SMC, and about 2 times greater than those measured with fibroblasts (Tables 1 and 2). Inclusion of glyburide in the initial incubation with unlabeled SMC inhibited the subsequent release of labeled FC from endothelial cells (Figure 5). In contrast, when SMC-conditioned medium was transferred to labeled endothelial cells, and glyburide added at that time, there was no inhibition of FC efflux from these cells. Vanadate added with apo A-1 to unlabeled SMC, and then removed by dialysis, did not reduce the rate of FC efflux from HAEC [4.8 ± 0.2 vs 5.0 ± 0.1 ng h^{-1} (μg of cell FC)$^{-1}$]. In contrast, when either vanadate or okadaic acid was added to SMC-conditioned medium in the presence of endothelial cells, FC efflux was strongly inhibited (Figure 5). The stimulation of FC efflux from HAEC by medium from control and FC-loaded SMC was equivalent.

To determine whether the stimulation of FC efflux from HAEC by SMC-conditioned medium depended solely on its PL content, PL sonicated vesicles were added in EBM in the absence of apo A-1 at a concentration of 4.6 μg/mL, the mean PL level assayed in conditioned medium after incubation for 5 h with SMC (92.4 × 5 × 10 ng/mL) (Table 1). The stimulation of FC efflux from HAEC over basal rates was <5% of that assayed under the same conditions with

SMC-conditioned medium containing apo A-1 (3 ± 1%, three experiments). To determine whether the stimulation of [^{3}H]FC efflux into conditioned medium reflected an exchange reaction, the experiment described above was repeated with PL vesicles containing FC at a concentration of 0.5 μg/mL, the mean FC level assayed in SMC-conditioned medium containing apo A-1 (12.8 × 5 × 10 ng/mL) (Table 2). Under these conditions, there was also no significant stimulation of [^{3}H]FC efflux from HAEC (1 ± 3% of the rates with conditioned medium, four experiments). [^{3}H]FC efflux from HAEC to FC/PL vesicles in the presence of vanadate (1 mM) was similar (3 ± 2% of the rates with conditioned medium).

These findings suggested that FC efflux from endothelial cells was promoted by apo A-1 only after this protein had first been primed with PL as the result of the ABC-1 transporter activity of SMC. More generally, they suggested that glyburide inhibited ABC-1-dependent PL efflux but that its effect on FC efflux was indirect, while vanadate inhibited FC efflux via a pathway distinct from that mediating the efflux of PL.

Effects of FC Preloading on SMC and Fibroblasts on the Properties of ABC-1 Activity. Preloading with FC significantly increased the level of expression of ABC-1 mRNA in fibroblasts and, more modestly, increased its already substantial level of expression in unloaded SMC. The effects of FC loading on PL and FC efflux, and on inhibition of these fluxes by glyburide and vanadate, were determined under the same conditions described above for unloaded cells.

Equilibration between cellular pools within [^{3}H]FC-labeled loaded cells was first determined by comparing the specific activity of total cell FC with that of FC effluxed into the medium. The medium FC specific activity was 0.98 ± 0.04 of that of the cell FC specific activity in the same experiment (n = 3). This result indicates full equilibration between cell surface and internal FC pools under the conditions used in the assay.

The effects of FC loading on PL and FC efflux were then determined. Compared to efflux in unloaded cells under the same assay conditions, PL efflux was enhanced (2.1 ± 0.4)-fold in SMC and (8.7 ± 0.2)-fold in fibroblasts (n = 3). This is comparable to the 2- and 10−12-fold increase observed in ABC-1 mRNA levels (Figure 4). Under the same conditions of FC loading, FC efflux was enhanced (1.8 ± 0.7)-fold in SMC and (14.0 ± 1.2)-fold in fibroblasts. In these experiments, FC efflux was inhibited 76 ± 6% by 1 mM vanadate and 79 ± 2% by 200 μM glyburide in unloaded SMC. It was inhibited 78 ± 6% by 1 mM vanadate and 81 ± 2% by 200 μM glyburide in loaded SMC (differences not significant). Comparable results were obtained with fibroblasts. These data indicate that the properties of lipid efflux from normal and FC loaded cells were similar.

DISCUSSION

Data from several studies now indicate that the promotion of lipid efflux to lipid-free apo A-1 involves the active participation of cell surface proteins. In the case of PL efflux, the essential role of ABC-1 transporter activity was recently identified (*16*−*19*) and is confirmed in the study presented here. The PL specificity of ABC-1-dependent transport has not been previously identified. A role for this transporter in transmembrane phosphatidylserine distribution was recently described (*23*). In our studies, little or no apo A-1-dependent movement of this lipid into the medium was detected. This suggests that the action of ABC-1 to redistribute phosphatidylserine to the exofacial leaflet of the bilayer does not involve transfer of this PL out of the membrane bilayer. This PL could increase the affinity of apo A-1 for the cell surface, either via protein−protein interaction (*19*) or by modifying the distribution of charged lipids on the cell surface (*23*). A similar indirect action for MDR-2, a related transporter, has been suggested (*36*). The major PL transported out of the cell in our studies was phosphatidylcholine. This is a major component of the exofacial leaflet of the membrane bilayer (*37*). In the case of fibroblasts but not of SMC, a significant amount of phosphatidylinositol was also recovered when apo A-1 was present in the medium. This difference, like the predominance of phosphatidylcholine in both cell lines, may reflect the lipid specificity of the ABC-1 transporter. However, in view of the otherwise similar properties of ABC-1 in the two cell types, it is more likely to reflect differences in the PL composition of the membrane domain where ABC-1 is localized at the surface of the plasma membrane.

ABC-1 transporter expression in SMC and endothelial cells has not previously been described, although the protein is widely distributed among tissues and cultured cell lines (*34*, *38*). Data presented here strongly suggest an almost complete absence of ABC-1 mRNA and activity in both HUVEC and HAEC. Unlike the situation in SMC and fibroblasts, expression was not upregulated when the cells were comparably FC-loaded. It had been reported previously that rat SMC were almost deficient in FC efflux to delipidated apo A-1, while the rate of PL efflux was similar to that of normal skin fibroblasts (*39*, *40*). Very recently, it was reported that human SMC, unlike the same cells in rodents, showed significant rates of FC efflux (*41*). The data in this research may help explain these disparate observations, by illustrating the independent regulation of PL and FC transport to apo A-1.

In contrast to current ideas on the function of the ABC-1 transporter, the study presented here strongly suggests that the transfers of PL and FC from the cell represent distinct pathways and that while PL efflux is directly dependent upon ABC-1 transporter activity, most FC efflux is not. The apparent K_m for FC efflux was significantly greater than that for PL. FC efflux was inhibited by okadaic acid and by vanadate, while PL efflux was not. Glyburide directly inhibited PL efflux, but did not reduce the rate of FC efflux when added with SMC-conditioned medium to endothelial cells. The effects of both okadaic acid and vanadate may be linked to the origin of FC in caveolae. Okadaic acid reduces the level of expression of caveolae at the cell surface by promoting surface flattening and redistribution of preformed caveolin to the intracellular pools (*42*). It also reduces transcriptionally the level of expression of caveolin, a major caveolar structural protein, by inhibiting formation of a nucleoprotein transcription complex containing transcription factors E2F and p53 (*43*). Vanadate is a specific inhibitor of P-type ATPases and of protein phosphotyrosine phosphatases (*26*, *44*). Ca^{2+}-ATPase, one of the major P-type ATPases of the plasma membrane, is localized almost exclusively within caveolae (*45*, *46*). In addition, it was recently shown that vanadate downregulated the expression

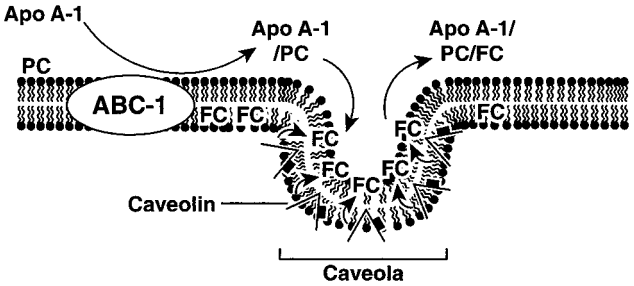

FIGURE 6: Model illustrating PL and FC efflux from the cell surface to apo A-1.

of caveolae at the cell surface (*47*, *48*). Under these conditions, phosphocaveolin accumulated intracellularly. Taken together, these data appear to be most consistent with the conclusion that the vanadate-sensitive component of FC efflux, representing approximately 80% of the total efflux from vascular cells, depends on one or more cell surface proteins other than ABC-1. This component of efflux appears to be distinct from simple diffusion. In erythrocytes, where FC efflux has been considered to represent diffusion (*33*), vanadate was without effect. This conclusion is also consistent with the inability of phosphatidylcholine vesicles to replace apo A-1−PL complexes in conditioned medium formed as the result of ABC-1 transporter activity. The finding that the effects of okadaic acid and vanadate on FC efflux were the same in fibroblasts and SMC (which express ABC-1 activity) and endothelial cells (which do not) is also consistent with the model of lipid efflux presented here. However, the possibility that a minor vanadate-resistant component of total FC efflux reflects cotransport with PL down the ABC-1-dependent pathway has not been ruled out by our experiments. Further research will also be required to establish additional details of the vanadate-dependent FC efflux pathway. Recent data indicate that several cell surface proteins which localize to caveolae, including SR-BI (*49*) and P-glycoprotein (*50*), may also be involved in FC homeostasis in different cells (*51*, *52*).

In summary, studies of the initial lipidation of apo A-1 in fibroblasts, SMC, and endothelial cells suggest that the efflux of PL and FC reflects different pathways for the most part and that PL efflux normally precedes the binding of FC. These steps are likely to have a significant influence on the conversion of lipid-free apo A-1 to HDL. A model illustrating this hypothesis is shown in Figure 6.

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