Membrane Lipid Domains Distinct from Cholesterol/Sphingomyelin-Rich Rafts Are Involved in the ABCA1-mediated Lipid Secretory Pathway*

Efflux of excess cellular cholesterol mediated by lipid-poor apolipoproteins occurs by an active mechanism distinct from passive diffusion and is controlled by the ATP-binding cassette transporter ABCA1. Here we examined whether ABCA1-mediated lipid efflux involves the selective removal of lipids associated with membrane rafts, plasma membrane domains enriched in cholesterol and sphingomyelin. ABCA1 was not associated with cholesterol and sphingolipid-rich membrane raft domains based on detergent solubility and lack of colocalization with marker proteins associated with raft domains. Lipid efflux to apoA-I was accounted for by decreases in cellular lipids not associated with cholesterol/sphingomyelin-rich membranes. Treating cells with filipin, to disrupt raft structure, or with sphingomyelinase, to digest plasma membrane sphingomyelin, did not impair apoA-I-mediated cholesterol or phosphatidylcholine efflux. In contrast, efflux of cholesterol to high density lipoproteins (HDL) or plasma was partially accounted for by depletion of cholesterol from membrane rafts. Additionally, HDL-mediated cholesterol efflux was partially inhibited by filipin and sphingomyelinase treatment. Apo-A-I-mediated cholesterol efflux was absent from fibroblasts with nonfunctional ABCA1 (Tangier disease cells), despite near normal amounts of cholesterol associated with raft domains and normal abilities of plasma and HDL to deplete cholesterol from these domains. Thus, the involvement of membrane rafts in cholesterol efflux applies to lipidated HDL particles but not to lipid-free apoA-I. We conclude that cholesterol and sphingomyelin-rich membrane rafts do not provide lipid for efflux promoted by apolipoproteins through the ABCA1-mediated lipid secretory pathway and that ABCA1 is not associated with these domains.

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Cellular cholesterol efflux occurs by at least two distinct mechanisms (reviewed in Refs. 1–3). Aqueous diffusion involves desorption of membrane cholesterol into the aqueous compartment surrounding cells followed by absorption to an appropriate acceptor, such as high density lipoproteins (HDL).1 Efflux by aqueous diffusion is independent of cell growth state, cholesterol content, and metabolic energy and does not involve active cellular transport pathways (4–8). This process is facilitated by the interaction of HDL particles with scavenger receptor B1 (SR-B1) (9, 10). A second pathway is mediated by lipid-free apolipoproteins and involves specific cellular events distinct from aqueous diffusion. Apolipoprotein-mediated cholesterol efflux is only apparent in growth-arrested or cholesterol-enriched cells, and it requires metabolic energy (5, 6, 11, 12) and a functional Golgi apparatus transport system (5–8). Apolipoprotein-dependent cholesterol efflux is absent in fibroblasts from patients with Tangier disease (TD), whereas efflux by aqueous diffusion mechanisms occurs normally in cells from affected individuals (8, 13, 14). Mutations in the gene encoding the ATP-binding cassette transporter ABCA1 are the underlying cause of TD (15–20), implicating this protein as a rate-controlling step in the apolipoprotein-mediated lipid efflux pathway. This conclusion was further supported by studies showing that the activity of the apolipoprotein-mediated lipid efflux pathway paralleled the level of expression of ABCA1 in cells (15, 18, 21). Although the mechanisms involved in cholesterol efflux are becoming better understood, there are limited data describing the cellular pools of lipids removed from cells by extracellular acceptors.

When cultured cells are incubated with whole plasma, cholesterol released from cells during early times (less than 30 min) preferentially associate with a minor subfraction of lipoproteins called pre-β1 HDL (22–24). These particles, composed mostly of apoA-I, may promote cholesterol efflux by a mechanism similar to that mediated by lipid-free apolipoproteins. Cell membrane caveolae domains may play a role in providing cholesterol to plasma acceptors during the initial efflux period (24, 25). Caveolin mRNA levels were increased in response to free-cholesterol enrichment of fibroblasts, and this was proportional to increased free cholesterol efflux promoted by HDL present in whole plasma (26, 27).

These observations raise the possibility that caveolae participate in delivery of intracellular lipids to the plasma membrane for removal by ABCA1-dependent efflux mechanisms. Caveolae are plasma membrane structures enriched in cholesterol and sphingomyelin and contain a variety of distinct proteins (reviewed in Refs. 28–30). Caveolae are one component of cellular membranes broadly termed membrane rafts, discreet microdomains present on the cell surface. Raft domains exist in cells

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1 The abbreviations used are: HDL, high density lipoprotein; SR-B1, scavenger receptor B1; TD, Tangier disease; TX-100, Triton X-100; DEMEM, Dulbecco’s modified Eagle’s medium; BSA, bovine serum albumin; 8-Br-cAMP, 8-bromo-cAMP; LDL, low density lipoprotein; MES, 4-morpholineethanesulfonic acid.
that do not express caveolin, and use of detergent-free systems have allowed the separation of caveolae from other raft microdomains (31, 32).

Simons and Ikonen (33) proposed the "raft hypothesis," in which sphingolipid:cholesterol microdomains are involved in numerous cellular functions, including membrane trafficking and signaling. According to this hypothesis, the lateral assembly of sphingolipids and cholesterol creates rafts floating in a glycerophospholipid-rich environment. These domains were first identified as membrane complexes insoluble in Triton X-100 (TX-100) at 4 °C (34, 35), and detergent insolubility has been used as a tool to identify lipid rafts and associated proteins (36). These TX-100-insoluble cellular membranes are enriched in glycosphingolipid and sphingomyelin and contain a significant fraction of the membrane cholesterol. In contrast, most of the cell glycosphingolipids are soluble in TX-100 (37, 38). The integrity of rafts depends on the presence of both cholesterol and sphingomyelin (39, 40).

In the current study, we examined whether ABCA1 was present in membrane rafts and if plasma membrane raft domains were involved in or required for ABCA1-mediated cholesterol and phospholipid efflux.

EXPERIMENTAL PROCEDURES

Cell Culture—All cell culture incubations were performed at 37 °C in a humidified 5% CO2 incubator. Human skin fibroblasts from patients having TD and from normal subjects were grown and maintained as described previously (7, 13, 41). Cellular cholesterol and choline-containing phospholipids were labeled as described previously (5). Labeled cells were cholesterol-enriched by incubation with DMEM containing 2 mg/ml fatty acid-free bovine serum albumin (BSA) and 30 cells were cholesterol-enriched by incubation with DMEM containing 2 mg/ml BSA (DMEM/BSA) to allow equilibration of cholesterol pools. For incubations with DMEM/BSA containing 1 mM 8-Br-cAMP as described below, fibroblasts were cholesterol-loaded and solubilized in MES-buffered saline containing 1% TX-100 and 1 mM phenylmethylsulfonyl fluoride (for lipid analysis, supernatants and pellets were extracted with CHCl3:CH3OH (3:2; v/v), and lipids were subjected to TLC as above to separate free and esterified cholesterol or phospholipids as indicated in individual experiments. For protein analyses, the pellet was suspended in TX-100 buffer containing HEPES (pH 7.4) instead of MES and incubated at room temperature for 30 min to solubilize rafts. Aliquots of the supernatant and solubilized pellet were used for immunoblot and ABCA1 immunoprecipitation.

Isolation of Membrane Rafts—Low density membrane rafts were separated from other cellular organelles by the sucrose density gradient method of Sargiacomo et al. (38). Briefly, cells grown in 150-mm dishes were cholesterol-loaded and solubilized in MES-buffered saline containing 1% TX-100 and 1 mM phenylmethylsulfonyl fluoride as above. The entire reaction was adjusted to 40% sucrose and placed in an ultra centrifuge tube. A 5–30% linear sucrose gradient was layered above the samples and centrifuged for 16 h at 39,000 rpm in a SW 41 rotor. After centrifugation, 1-mL fractions were collected from the top of the tube for [3H]cholesterol and caveolin-1 determinations.

Membrane Rafts and ABCA1-mediated Lipid Efflux

Cholesterol esterification was expressed as nanomoles of [14C]oleate incorporated into [14C]cholesterol esters per milligram of cell protein.

Triton X-100 Cell Solubilization—Cells were separated into TX-100-soluble and -insoluble fractions by published methods (37). Confluent cell monolayers were washed in phosphate-buffered saline (PBS) and then incubated in MES-buffered saline (25 mM MES, pH 6.5, 0.15 mM NaCl) containing 1% Triton X-100 and 1 mM phenylmethylsulfonyl fluoride (for lipid analysis) and 1 μM leupeptin, 1 μM pepstatin, and 1 μM aprotinin (for protein analysis). The suspension was homogenized by 10 strokes with a Dounce homogenizer, kept on ice for 20 min, and centrifuged at 14,000 × g for 20 min at 4 °C. The supernatant (containing the Triton X-100 fraction) was removed. For lipid analysis, supernatants and pellets were extracted with CHCl3:CH3OH (3:2; v/v), and lipids were subjected to TLC as above to separate free and esterified cholesterol or phospholipids as indicated in individual experiments. For protein analyses, the pellet was suspended in TX-100 buffer containing HEPES (pH 7.4) instead of MES and incubated at room temperature for 30 min to solubilize rafts. Aliquots of the supernatant and solubilized pellet were used for immunoblot and ABCA1 immunoprecipitation.

Media, Lipoproteins, and Apolipoproteins—Blood was collected into vacuum tubes containing streptokinase (180 units/ml final concentration) and chilled on ice, and plasma was separated by centrifugation (2000 × g for 20 min). Plasma was used for experiments within 60 min of isolation.

LDL and HDL were prepared from pooled EDTA plasma by ultracentrifugation in the density intervals 1.019–1.063 and 1.12–1.21 g/ml, respectively. HDL was depleted of apoE- and apoB-containing particles by heparin-agarose chromatography (7). LDL was acetylated by the method of Goldstein et al. (42). ApoA-I was purified from HDL as described previously (43). Protein was measured by the method of Goldstein and Goldstein (44). The integrity of rafts was maintained by the use of detergent-free systems (34, 35), and detergent insolubility has been used as a tool to identify lipid rafts and associated proteins (36). These TX-100-insoluble cellular membranes are enriched in glycosphingolipid and sphingomyelin and contain a significant fraction of the membrane cholesterol. In contrast, most of the cell glycosphingolipids are soluble in TX-100 (37, 38). The integrity of rafts depends on the presence of both cholesterol and sphingomyelin (39, 40).

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Metabolic Labeling and Cell-Surface Biotinylation—Cellular proteins were labeled with [35S]methionine overnight in the equilibration medium. The different incubation conditions used had no effect on total cell [35S]Protein-specific activity. For selective labeling of plasma membrane proteins, fibroblasts were incubated for 30 min at 0 °C with phosphate-buffered saline containing 1 mg/ml sulfo-N-hydroxysuccinimide-biotin to biotinylate cell-surface proteins (45). Cells were then solubilized in detergent, and ABCA1 was isolated by immunoprecipitation as described below.

Immunoprecipitation—Rabbit antiserum for ABCA1 was raised against a synthetic peptide located at the COOH terminus of human ABCA1 (18). This antibody had immunoprecipitating activity but was ineffective on immunoblots (18). For immunoprecipitation, TX-100 extracts were incubated overnight at 4 °C with the ABCA1 antiserum (1:200 dilution) followed by an additional 1-h incubation with protein G-coated magnetic beads (Zymed Laboratories Inc., laboratories, San Francisco, CA). Lake Success, NY). The antibody-protein antigen complex was sedimented with a magnet, the beads were washed twice with TX-100 buffer, and proteins were eluted with 1% acetic acid. After neutralization with 1 M Tris (pH 7.4), proteins were solubilized in SDS buffer for electrophoresis.

SDS Polycrylamide Gel Electrophoresis and Immunoblotting—Proteins were solubilized in 50 mM Tris buffer containing 1% SDS, 0.1 mM phenylmethylsulfonyl fluoride, and reduced by boiling in SDS-sample gel electrophoresis using 6 and 15% gels for ABCA1 and caveolin-1, respectively. For each cell extract, the volume of SDS buffer added per lane represented the fraction of protein partitioning between TX-100-soluble and -insoluble membranes. To ensure that the same amount of total cell protein was represented for each experimental condition and cell line, aliquots of the initial TX-100 extracts were assayed for protein content, and the volume of SDS buffer applied to each lane was adjusted accordingly. Immunoprecipitated [35S]methionine-labeled ABCA1 was visualized in dried gels by phosphorimaging. For immunoblots, proteins were transferred to nitrocellulose membranes and probed with a 1:5000 dilution of rabbit antiserum to human caveolin-1 (Transduction Laboratories, Lexington, KY). 0.25 μg/ml mouse IgG to human clathrin heavy chain (Transduction Laboratories), 1 μg/ml mouse IgG to human Transferrin Receptor (Zymed Laboratories Inc. Laboratories, San Francisco, CA), or 2 μg/ml rabbit IgG to human T-cadherin (raised against the 15 amino acids of the NH2 terminus of the mature protein). Antibody-positive bands were visualized by an enhanced chemiluminescence assay (ECL, Amersham Pharmacia Biotech, Piscataway, NJ). For detection of biotinylated ABCA1, proteins were transferred to nitrocellulose and visualized with a streptavidin-horseradish peroxidase ECL assay.

Other Methods—Lactate dehydrogenase in cell culture medium or in cell layers solubilized with 1% TX-100 at room temperature was measured using a commercial kit (Roche Diagnostics, Indianapolis, IN). Statistical comparisons were made by paired Student’s t test with significance assumed for p values less than 0.05. All results were confirmed in
ter detergent solubilization. Similar to 35S-labeling studies, we approached cell-surface proteins were biotin-labeled prior to de-T fibroblasts, as reported previously (18). For the second induced cells compared with controls in both the normal and TX-100-soluble fraction. ABCA1 levels markedly increased in blasts were incubated for 48 h with DMEM containing 2 mg/ml BSA and DMEM containing 1 mg/ml BSA. To induce ABCA1 expression, fibroblasts were incubated for 48 h with DMEM containing 2 mg/ml BSA and 30 μg/ml cholesterol followed by 24-h incubations with DMEM containing 1 mg/ml BSA and 1 mM 8-Br-cAMP. Total cell proteins were labeled by including [35S]methionine in the medium during the final 24-h incubations (20S-ABCA1). Cell surface proteins were labeled by with sulf-o-N-hydroxysuccinimide-biotin (bt-ABCA1). After appropriate pre-treatment, the TX-100-soluble (+) and -insoluble (-) proteins were isolated. Immunoprecipitated [35S]ABCA1 and immunodetectable cavelin-1 (CAV1) and clathrin (CLATH) were identified as described under “Experimental Procedures.”

at least two independent experiments.

RESULTS

Distribution of ABCA1 between TX-100-soluble and -insoluble Membranes—ABCA1 plays a critical role in apolipoprotein-mediated cholesterol efflux (15–20), perhaps by transporting cellular lipids across the plasma membrane to apolipoproteins and/or shuttling lipids between other cellular compartments and the plasma membrane. We examined whether ABCA1 was associated with cholesterol and sphingomyelin-rich membrane raft domains. For these studies we used immortalized normal and TD fibroblast lines shown to exhibit massive increases in ABCA1 expression in response to cholesterol loading and 8-Br-cAMP treatment (18). The fractional distribution of cholesterol and phospholipids in membrane rafts in these cells is identical to that in the parental primary cell lines (41). Cells were maintained in growth medium containing serum (controls) or were induced by cholesterol and 8-Br-cAMP treatment, and the distribution of ABCA1 and domain-specific membrane proteins between TX-100-soluble and -insoluble fractions was examined. The TX-100-insoluble fraction in both control and treated cells contained ~60% of the total sphingomyelin and less than 10% of the phosphatidylcholine, consistent with the properties of membrane rafts (37–40).

We took two approaches to identify ABCA1. First, cellular proteins were labeled with [35S]methionine, and ABCA1 was immunoprecipitated from each fraction. By this method, nearly all of the detectable 35S-labeled ABCA1 was present in the TX-100-soluble fraction. ABCA1 levels markedly increased in induced cells compared with controls in both the normal and TD cells (Fig. 1), although the levels were significantly lower in TD fibroblasts, as reported previously (18). For the second approach, cell-surface proteins were biotin-labeled prior to detergent fractionation and ABCA1 was immunoprecipitated after detergent solubilization. Similar to 35S-labeling, studies, levels of ABCA1 were increased by cholesterol and 8-Br-cAMP treatment, and all detectable biotin-labeled ABCA1 protein was present in the TX-100-soluble (i.e. nonraft) membrane fraction (Fig. 1). The distribution of other membrane proteins was also examined. In contrast to ABCA1, the vast majority of cavelin-1 was present in the detergent-insoluble or membrane raft fraction of both normal and TD cells, indicating a distinct localization compared with membranes containing ABCA1 (Fig. 1). The relative immunoreactivity of cavelin-1 was not markedly different between the immortalized normal and TD cells incubated without or with cholesterol and 8-Br-cAMP.

To further verify that TX-100 solubility provided separation of membrane rafts from other membranes, the distribution of additional marker proteins were also examined by immunoblotting. The GPI-anchored protein t-cadherin, shown to localize to noncaveolae membrane raft domains (49, 50), had the same membrane distribution as cavelin-1 (not shown). In contrast, clathrin (Fig. 1) and the transferrin receptor (not shown), associated with membranes distinct from membrane rafts, (50, 51) were present in the TX-100-soluble (nonraft) membrane fraction. These data indicate that the TX-100 solubility of cell membranes under the described condition separated membrane raft-associated proteins from nonraft-associated proteins.

Cholesterol Efflux from TX-100-soluble and -insoluble Membranes—To assess which cellular pool(s) provide(s) cholesterol for efflux, we incubated [3H]cholesterol-labeled and cholesterol-enriched fibroblasts with purified apoA-I, isolated HDL, or plasma. After incubation, cells were homogenized with TX-100 buffer, and the detergent-soluble and -insoluble fractions were separated. Changes in cholesterol label and mass in each fraction were compared relative to cells incubated without cholesterol acceptors (Fig. 2). In the control cells 32 ± 4% and 38 ± 2% of the [3H]cholesterol and cholesterol mass were recovered in the TX-100-insoluble fraction, respectively. The detergent-insoluble fraction contained about 20% of total cellular protein. The TX-100-insoluble fraction was significantly enriched in cholesterol compared with the TX-100-soluble fraction when normalized for protein, 253 ± 16 and 61 ± 2 μg of cholesterol/mg of protein, respectively. Efflux of cholesterol mediated by apoA-I was accounted for by decreased free cholesterol in the TX-100-soluble (i.e. nonraft) cell fraction without significant change in the TX-100-insoluble (raft) fraction assessed by changes in radiolabel or mass. In contrast, cells incubated with plasma or HDL showed a significant decrease of [3H]cholesterol in both the TX-100-soluble and -insoluble fractions. Cholesterol efflux (label or mass) was greatest for plasma followed by HDL then apoA-I. Approximately 30% of the decrease in cell cholesterol mediated by HDL or plasma was due to depletion of cholesterol from the TX-insoluble fraction. However, when plasma was used as the acceptor, the change in cholesterol mass was underestimated as compared with efflux of [3H]cholesterol. This apparent discrepancy could be accounted for by a 15 and 11% decrease in the specific activity of labeled cholesterol in the TX-100-insoluble and -soluble fractions, respectively, after incubation with plasma, and most likely accounted for by the exchange of cholesterol by diffusional pathways. In contrast, apoA-I and HDL had no significant effect on the specific activity of cellular [3H]cholesterol. These data show that efflux mediated by apoA-I was limited to removal of cholesterol from membranes distinct from cholesterol and sphingomyelin-rich rafts. In contrast, HDL and acceptors present in whole plasma could also mediate the depletion of cholesterol associated with membrane rafts.

In a similar study, we examined apoA-I-mediated efflux of phospholipids from cholesterol-enriched cells (Fig. 3). A decrease in the TX-100-soluble fraction accounted for apoA-I-mediated efflux of radioactive phosphatidylcholine and sphingomyelin (although the sphingomyelin decrease did not reach significance), and there was no change in the phospholipid content of the TX-100-insoluble fraction. These results are con-
sistent with the notion that cellular lipids removed by apoA-I arise from membrane fractions with detergent solubility properties distinct from membrane rafts.

Efflux Studies in TD Fibroblasts—Fibroblasts from patients with TD lack the apolipoprotein-mediated cholesterol efflux pathway (13) due to mutations in the gene for ABCA1 (15–20), indicating that this transporter has a direct role in modulating apolipoprotein-mediated lipid efflux. We examined whether defective ABCA1 function in TD produced alterations in the properties of membrane rafts or affected efflux by other pathways. The distribution of [3H]cholesterol between TX-100-soluble and -insoluble fractions and pools accessible to cholesterol oxidase was compared in TD and normal fibroblasts after incubation with acceptors (Fig. 4). Cholesterol accessible to oxidation by exogenous cholesterol oxidase is believed to be localized to caveolae (24, 52). Fibroblasts from TD patients had similar proportions of [3H]cholesterol present in TX-100-insoluble fraction as did normal cells (34.2 ± 4.8 versus 37.1 ± 2.5%, respec-
with \[^{3}H\]cholesterol and cholesterol-loaded as described under “Exper-

Normal and TD fibroblasts were radiolabeled and TD fibroblasts.

accessible to cholesterol oxidase in cholesterol-loaded normal

four to eight dishes.

\[^{3}H\]cholesterol appeared as described under “Experimental Procedures.” Each value is the mean ± S.D. of four to eight dishes.

converted to \[^{3}H\]cholestenone, respectively, was measured as described under “Experimental Procedures.” Each value is the mean ± S.D. of four to eight dishes.

cholesterol oxidase accessibility of the remaining cellular labeled cholesterol to assess whether this pool of cholesterol was preferentially depleted from cells.

In cells not enriched with cholesterol, apoA-I-mediated cholesterol efflux was not detected from control or TD cells as expected (not shown). When 5% human plasma was present in the culture medium, appreciable \[^{3}H\]cholesterol appeared in the medium. The majority of the cholesterol efflux was accounted for by a decrease in the cholesterol oxidase-resistant cholesterol, but in both cell lines oxidase-sensitive cholesterol (i.e. caveolae-associated) decreased significantly. In cholesterol-enriched cells, efflux to plasma in both cell lines was again associated with a decrease in both cholesterol oxidase-resistant and -sensitive pools. Because efflux of radiolabeled cholesterol was monitored, the change in cell \[^{3}H\]cholesterol could be due to exchange without changes in cell mass. Nevertheless, caveolae cholesterol content (based on cholesterol oxidase sensitivity) was affected in both cell lines to a similar extent when incubated with whole plasma. Similar results were obtained when isolated HDL was used as the acceptor (not shown). There was no apoA-I-mediated efflux activity in TD cells, as expected (15, 16), and no difference in cholesterol distribution after incubation with apoA-I. In contrast, apoA-I effectively promoted cholesterol efflux and decreased cholesterol present in the cholesterol oxidase-resistant (non-caveolae) pool without affecting the cholesterol oxidase-sensitive pool in the normal cells. Thus, efflux of cholesterol promoted by apoA-I was accounted for by a decrease in cholesterol not associated with caveolae. Furthermore, serum- and HDL-mediated efflux of caveolae cholesterol occurred similarly in normal and TD cells, suggesting that efflux of cholesterol from these sites occurs independently of a functional ABCA1 protein.

Filipin Treatment—Filipin binds to cholesterol-rich domains of cellular membranes and disrupts raft structure and organization (53–57). We examined the effects of treating cells with filipin on \[^{3}H\]cholesterol efflux from cholesterol-enriched fibroblasts by apoA-I or HDL (Fig. 6). Filipin had no effect on the recoveries of cell protein or \[^{5}H\]cholesterol or release of lactate dehydrogenase (as a measure of cell permeability and viability) compared with controls (not shown). Filipin treatment had no significant effect on cholesterol efflux to control medium containing only albumin or the extent of efflux mediated by apoA-I.
Cells were labeled with [3H]cholesterol as described under “Experimental Procedures” then received medium with DMEM containing 2 mg/ml BSA with either 0 or 30 μg/ml cholesterol (Chol pre-treat) for 24 h. Cells receiving excess cholesterol were incubated an additional 48 h to equilibrate cholesterol pools. Cultures were then incubated for 6 h with DMEM containing 1 mg/ml BSA (DMEM) or the same medium containing 5 μg/ml apoA-I or 5% plasma (total cholesterol = 189 mg/dl). After incubation, medium was removed; cells were rinsed twice with PBS then incubated for 30 min in PBS containing 0.5 units/ml cholesterol oxidase. Cell layers were extracted, and lipids were subjected to TLC to separate cholesterol (Oxidase-resistant [3H]Chol), cholesterol ester (Oxidase-sensitive [3H]Chol), and cholesterol esters ([3H]Chol esters) for quantitation of radioactivity. Results are expressed as the percentage of total [3H] and are the means ± S.D. of three dishes.

### TABLE I

**Efflux and cholesterol (Chol) oxidase sensitivity of [3H]cholesterol in Tangier disease and normal fibroblasts**

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Chol pre-treat</th>
<th>Efflux medium</th>
<th>Chol efflux</th>
<th>Oxidase-resistant [3H]Chol</th>
<th>Oxidase-sensitive [3H]Chol</th>
<th>Cell [3H]Chol esters</th>
<th>% of total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tangier disease</td>
<td>0 μg/ml</td>
<td>DMEM</td>
<td>4.6 ± 1.3</td>
<td>84.1 ± 1.6</td>
<td>10.6 ± 0.4</td>
<td>0.8 ± 0.1</td>
<td></td>
</tr>
<tr>
<td>30 μg/ml</td>
<td>5% plasma</td>
<td>DMEM</td>
<td>19.4 ± 2.8^a</td>
<td>71.9 ± 3.8^a</td>
<td>6.9 ± 0.5^a</td>
<td>1.8 ± 0.4^a</td>
<td></td>
</tr>
<tr>
<td></td>
<td>apoA-I</td>
<td>5.9 ± 0.4</td>
<td>56.9 ± 2.2</td>
<td>21.3 ± 0.8</td>
<td>15.9 ± 1.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td>0 μg/ml</td>
<td>DMEM</td>
<td>3.7 ± 0.9</td>
<td>82.1 ± 1.8</td>
<td>12.4 ± 1.1</td>
<td>1.9 ± 0.2</td>
<td></td>
</tr>
<tr>
<td>30 μg/ml</td>
<td>5% plasma</td>
<td>DMEM</td>
<td>24.3 ± 1.4^a</td>
<td>64.2 ± 0.0^a</td>
<td>9.3 ± 1.1^a</td>
<td>2.2 ± 0.4</td>
<td></td>
</tr>
<tr>
<td></td>
<td>apoA-I</td>
<td>11.2 ± 0.4^a</td>
<td>34.8 ± 0.9^a</td>
<td>37.2 ± 0.8</td>
<td>16.8 ± 0.7</td>
<td></td>
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</tr>
</tbody>
</table>

*Indicates p < 0.05 by Student’s t test compared to same cells incubated with DMEM.

The lack of an effect of filipin suggests that apoA-I does not require structurally intact rafts to promote cholesterol efflux. Efflux of cellular [3H]cholesterol by HDL was decreased by ~25% (p < 0.05 at all doses of filipin) compared with control incubations. Decreased HDL-mediated efflux after filipin treatment could be due to depletion of cholesterol associated with membrane rafts. This possibility is supported by the observation that the decrease in efflux was similar to the proportion of efflux accounted for by cholesterol associated with membrane raft domains (i.e. TX-100-insoluble fraction, Fig. 2). These findings again show that, in contrast to lipid-free apoA-I, HDL can promote efflux of [3H]cholesterol associated with membrane rafts. These results, however, may be confounded by the fact that filipin will bind to HDL lipids and that such interactions may interfere with the ability of HDL to accommodate cholesterol.

**Sphingomyelinase Treatment**—A majority of membrane sphingomyelin is associated within raft domains in the plasma membrane (34). We reasoned that treating cells with sphingomyelinase would alter raft structure or composition and disrupt apolipoprotein-mediated lipid efflux from these domains, if they were involved in this process. The effect of sphingomyelinase treatment on apoA-I-mediated efflux of cellular [3H]-phospholipids was examined (Fig. 7). Sphingomyelinase treatment digested greater than 80% of cellular [3H]sphingomyelin without affecting levels of phosphatidyl[3H]choline (not shown). As expected, lipase treatment nearly abolished sphingomyelin efflux from cells. Loss of membrane sphingomyelin, presumed to be at least partially associated with membrane raft domains, had no appreciable effect on the ability of apoA-I to promote efflux of [3H]cholesterol and slightly increased phosphatidyl[3H]choline efflux. Cellular cholesterol esterification by acyl-CoA:cholesterol O-acyltransferase was measured in the same cells after the cholesterol efflux incubation. Acyl-CoA:cholesterol O-acyltransferase activity was significantly (p < 0.05) increased in sphingomyelinase-treated cells compared with controls, demonstrating the internalization of membrane cholesterol to acyl-CoA:cholesterol O-acyltransferase accessible pools by lipase action, as shown previously (58, 59). However, incubation with apoA-I decreased cholesterol esterification in both control and sphingomyelinase-treated cells, and the absolute decrease was similar for both conditions (a decrease of 1.2 ± 0.1 and 1.5 ± 0.3 nmol of cholesterol ester/mg of cell protein for control and treated cells, respectively). In the same study, cholesterol efflux by HDL decreased in sphingomyelinase-treated cells compared with controls, from 9.8 ± 0.6% to 7.9 ± 0.9% of total cell cholesterol. HDL decreased cholesterol esterification to a similar extent as apoA-I, and the extent of the decrease was not affected by sphingomyelin treatment (not shown) as occurred during incubations with apoA-I.

These results suggest that digestion of sphingomyelin depleted the pool of cholesterol associated with membrane rafts available for efflux by HDL without affecting cholesterol available for efflux by apoA-I, similar to results obtained using filipin-treated cells. Because the lipase was present during the incubation with HDL and cells, we cannot rule out the possibility that the decreased efflux was due to a direct effect on HDL composition affecting the ability to promote cholesterol efflux.

**DISCUSSION**

HDL components can remove cholesterol from cells by at least two distinct mechanisms. First, HDL phospholipids promote efflux of plasma membrane cholesterol by a passive diffusion process that is facilitated by HDL binding to SR-BI (9, 10) and suggested to involve caveolae (60). Second, lipid-poor HDL apolipoproteins remove excess cellular cholesterol by an
active, Golgi-dependent pathway (5–7) that is severely impaired in TD (13) and depends on a functional ABCA1 protein (15, 18, 20, 61). Although passive diffusion mechanisms may be significant in some cells, the massive accumulation of cholesterol in macrophages of TD patients demonstrates the physiological importance of the ABCA1 pathway in clearing cholesterol from macrophages. Previous studies showed that the ability of lipid-free apoA-I to promote cholesterol efflux from cells is independent of the level of SR-B1 expression (9) and that expression of SR-B1 can inhibit the extent of apoA-I-mediated lipid efflux (62), suggesting that this receptor does not play a role in the ABCA1-mediated lipid secretory pathway. Here we show that the ABCA1 efflux pathway also does not involve cholesterol and sphingomyelin-rich raft membrane domains.

Several lines of evidence support the lack of involvement of membrane rafts in the apolipoprotein-mediated lipid removal pathway. First, ABCA1 was associated with a membrane fraction distinct from cholesterol- and sphingomyelin-rich rafts. Second, efflux of lipids from cultured fibroblasts by apoA-I was accounted for by decreases in cellular lipids not associated with raft domains as defined by both detergent insolubility and accessibility to cholesterol oxidase. Third, apoA-I-mediated cholesterol efflux was completely absent from cultured TD fibroblasts despite normal amounts of raft lipids. Fourth, treating cells with filipin, which disrupts raft structure in other cell types (53–57), had no effect on apoA-I-mediated cholesterol efflux. In contrast, filipin treatment partially inhibited HDL-mediated cholesterol efflux, possibly by interfering with diffusional efflux of cholesterol associated with membrane raft domains. Fifth, sphingomyelinase treatment, which depleted over 80% of the cellular sphingomyelin, had no inhibitory effect on apoA-I-mediated cholesterol and phosphatidylcholine efflux. Because sphingomyelinase is required to maintain the integrity of rafts (39, 40), these results provide additional evidence that disrupting these structures does not impair the ABCA1 lipid secretory pathway. Interestingly, these data also showed that apoA-I-mediated lipid efflux can occur without the concomitant efflux of cellular sphingomyelin. These data imply that removal of lipids from membrane raft domains is not involved in apolipoprotein-mediated cholesterol efflux.

Mutations in ABCA1 account for the severe impairment of this pathway in TD and other familial HDL deficiencies (15, 18, 20, 61). We also show that compartments distinct from membrane raft domains contain all of the cellular and plasma membrane ABCA1, the rate-controlling protein in the apolipoprotein-mediated lipid removal pathway. As shown previously, treatment of immortalized normal fibroblasts with cholesterol and a cAMP analog, which enhances apoA-I-mediated lipid efflux, markedly induced ABCA1 expression and incorporation into the plasma membrane (18, 41). ABCA1 was present only in membranes soluble in TX-100, whether expressed at low or high levels. This membrane fraction contained nearly all of the phosphatidylcholine and nonraft protein markers (clathrin and the transferrin receptor), and it excluded most of the sphingomyelin and nearly all of the marker for caveolae (caveolin) and for noncaveolar rafts (T-cadherin) (49). Thus, these ABCA1-associated membranes do not contain significant amounts of caveolae or other types of sphingomyelin-rich rafts. ABCA1 expressed by one TD cell line, which contains a single amino acid substitution (15, 18), also localized to these nonraft membranes. Although this mutation nearly abolishes apoA-I-mediated lipid efflux (13, 41) and impairs ABCA1 expression (18), the processing and membrane distribution of this protein does not appear to be grossly defective in these cells.

It is noteworthy that caveolin-1 expression was not markedly affected by cholesterol and cAMP or the mutation in ABCA1, providing further evidence for unrelated functions of these two proteins. Based on accessibility to cholesterol oxidase, however, TD cells appeared to have below normal levels of caveolae cholesterol, suggesting that ABCA1 may have some effect on caveolae composition.

The precise function of ABCA1 is still unknown, but the structural similarities with other ABC transporters suggest a role in facilitating transmembrane movement of lipids. Its appearance on the cell surface supports the possibility that it may transport cholesterol and phospholipid directly to cell-surface-bound apolipoproteins, and this possibility is strengthened by recent reports showing that ABCA1 is an apoA-I-binding protein (63, 64). Whether ABCA1 also participates in intracellular lipid transport pathways leading to efflux independent of apolipoprotein binding cannot be excluded. Whatever the mechanism, our data indicate that ABCA1-mediated lipid secretion does not require membrane rafts.

Evidence that caveolae, a specialized type of membrane raft domain, play a role in cellular cholesterol transport and efflux comes from several studies showing that caveolin modulates sterol trafficking in cells and that HDL can selectively remove cholesterol from caveolar membranes. Caveolin mediates transport of newly synthesized cholesterol from the endoplasmic reticulum to caveolae (65) by a Golgi-independent process.
with pre-apoA-I with fibroblasts leads to the formation of HDL particles participating.

surface, a process in which lipid-free apoA-I does not decrease in raft cholesterol. In addition, HDL and plasma re-
ed cholesterol, and the free cholesterol content of cells regulated caveolin mRNA levels (26, 27). The observation that free cholesterol, and newly synthesized sterol was selectively en-
chelled by cholesterol from other cellular pools. This seems un-
currently depleted by incubations with plasma or HDL, suggest-
ing lipids as their major constituent. Further studies are needed to elucidate the nature of the different cholesterol efflux pathways, their contributions to cellular cholesterol homeosta-
sis, and their roles in depletion of excess cellular cholesterol that accumulates during atherogenesis.

REFERENCES


Membrane Rafts and ABCA1-mediated Lipid Efflux

Membrane Lipid Domains Distinct from Cholesterol/Sphingomyelin-Rich Rafts Are Involved in the ABCA1-mediated Lipid Secretory Pathway

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