Cellular Phospholipid and Cholesterol Efflux in High-Density Lipoprotein Deficiency

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Background—Prospective studies have examined the relationship between coronary artery disease and low plasma levels of high-density lipoprotein cholesterol (HDL-C).

Methods and Results—We investigated the causes of hypoalphalipoproteinemia (HypoA; HDL-C <5th percentile) in 64 subjects (12 women and 52 men). Apolipoprotein AI-mediated cellular cholesterol and phospholipid efflux were measured in fibroblasts from HypoA subjects, 9 controls, 2 patients with Tangier disease, and 5 patients with hyperalphalipoproteinemia. A phospholipid efflux defect was defined as <70% of controls. Mean HDL-C was 0.49±0.21 mmol/L. Cholesterol and phospholipid efflux correlated strongly (r = 0.72, P < 0.001). Phospholipid efflux and HDL-C (r = 0.64, P < 0.001) correlated in HypoA subjects. However, phospholipid or cholesterol efflux was no longer a determinant of HDL-C levels at higher levels (> 1.0 mmol/L) of HDL-C. In HypoA subjects, 4 cases of Tangier disease and 6 of familial HDL deficiency (heterozygous Tangier disease) were identified (10 of 64; 16%). In the remaining 54 subjects, mean lipid efflux was not significantly different from controls and subjects with hyperalphalipoproteinemia. A phospholipid efflux defect was identified in 7 additional HypoA subjects, and a cholesterol efflux defect was detected in 11 subjects. In 2 of these subjects, the ABCA1 gene was ruled out as the cause of the efflux defect, while in 3, the low HDL-C trait segregated with the ABCA1 gene locus.

Conclusions—Lipidation of lipid-poor apolipoprotein AI may not be a major determinant of cholesterol accumulation within more mature HDL particles and increasing cholesterol or phospholipid efflux beyond normal levels may not lead to increase in plasma HDL-C levels. ABCA1 is essential in the initial steps of HDL formation but other plasma events are major modulators of HDL-C levels. (Circulation. 2003;107:1366-1371.)

Key Words: lipoproteins ■ cholesterol ■ genetics ■ risk factors

The relationship between coronary artery disease and low plasma levels of high-density lipoprotein cholesterol (HDL-C) has been shown in prospective epidemiological studies. An HDL-C <1.0 mmol/L (40 mg/dL) is considered a categorical cardiovascular risk factor. The mechanisms by which HDL prevent atherosclerosis are multiple and include reverse cholesterol transport and improvement of vascular endothelial function. A low HDL-C is seen in 30% to 50% of cases of premature coronary disease. In most patients, low HDL-C is due to hepatic oversecretion of triglycerides and apolipoprotein (apo) B. A low HDL-C is also a prominent feature of familial combined hyperlipidemia and the metabolic syndrome. Some patients have isolated HDL-C deficiency that is often familial in nature. We have described such an entity, familial HDL deficiency (FHD), which is defined as HDL-C <5th percentile for age and sex and a decrease in apoAI-mediated cellular cholesterol efflux of 50% of normal. In fibroblasts from patients with Tangier disease (TD), apoAI-mediated cholesterol efflux is decreased to 10% of controls. FHD patients have monoallelic mutations at the ABCA1 gene locus (heterozygous TD). The ABCA1 gene product is thought to promote the transfer of cellular phospholipids and cholesterol onto an extracellular acceptor particle consisting of lipid-poor apoAI.

The present study was undertaken to determine the prevalence of cellular lipid efflux defects in subjects with severe hypoalphalipoproteinemia (HypoA), the prevalence of mutations at the ABCA1 gene locus in such patients, and to examine the relationship between cellular cholesterol efflux and phospholipid efflux. We also examined the role of cellular lipid efflux as a determinant of plasma HDL-C levels.

Methods

Patient Selection

Patients were selected from the McGill University Health Center, Montreal, Canada, and the Academic Medical Center of Amsterdam, the Netherlands. The research protocol was reviewed by the Ethics Committees of each institution. Informed consent was required for blood sampling, DNA analysis, and fibroblast cultures. Patients with HDL-C <5th percentile for age and sex-matched subjects (HypoA;
Chloroform:methanol (2:1). Efflux was determined as percent of total phospholipids in the medium. All results were confirmed at least twice. Because of the possibility of phospholipid hydrolysis after a prolonged incubation, phospholipid efflux was also performed after a 16-hour ³H-choline labeling protocol. Final results did not differ from longer incubation (data not shown). The linearity of the efflux experiments was verified at time points 0, 2, 4, 8, 12, and 24 hours in cell lines from normal, HyperA, HypoA, FHD, and TD subjects.

### Separation of Lipoproteins by 2D Gel Electrophoresis
Plasma lipoproteins were separated by 2D gel electrophoresis as described previously. After electrophoresis, lipoproteins were transferred (30 V, 4 h) to a nitrocellulose membrane (Hybond ECL, Amersham). Immunolocalization of apoAI to a nitrocellulose membrane was performed by using immunopurified anti-human apoAI antibody (Biodesign International).

### Cell Culture
Skin fibroblast cultures were established from biopsies of the anterior forearm. Efflux experiments were performed on all subjects and controls.

### Cholesterol Efflux
We seeded 50×10⁶ cells in 12-well plates. The efflux protocol for efflux studies has been previously described. Efflux was determined as percent of total ³H-cholesterol measured in the medium, using apoAI (10 μg/mL) as acceptor. All experiment results were confirmed at least 3 times.

### Phospholipid Efflux
Using a similar protocol, 50×10⁶ cells were seeded in 12 well plates. The cells were labeled with 1 μCi/mL ³H-choline for 72 hours. At confluence, cells were cholesterol-loaded (20 μg/mL) as above. After a 24-hour equilibration period, phospholipid efflux was determined at 24 hours with 10 μg/mL apoAI. Efflux medium was collected, and cellular lipids were extracted with hexane/isopropanol (3:2), while total phospholipids from the medium were extracted in chloroform:methanol (2:1). Efflux was determined as percent of total ³H-phospholipids in the medium. All results were confirmed at least twice. Because of the possibility of phospholipid hydrolysis after a prolonged incubation, phospholipid efflux was also performed after a 16-hour ³H-choline labeling protocol. Final results did not differ from longer incubation (data not shown). The linearity of the efflux experiments was verified at time points 0, 2, 4, 8, 12, and 24 hours in cell lines from normal, HyperA, HypoA, FHD, and TD subjects.

### Results

#### Study Subjects
We studied 80 subjects; 9 controls with a mean plasma HDL-C of 1.30±0.22 mmol/L, 2 TD subjects, and 5 subjects with HyperA (HDL-C >90th percentile) were used as controls for the cellular lipid efflux experiments. In the present study, we examined 64 patients with plasma HDL-C <5th percentile for age and sex. We excluded family members so that no familial relationship is known to occur between cases. Of the 64 patients, 36 came from Canada (province of Quebec, n=34, and British Columbia, n=2) and 26 patients were referred from the Netherlands. Referrals from the Czech Republic (n=1) and Japan (n=1) were performed for evaluation of TD. There were 52 men and 12 women. The mean age was 49±12 years. The mean total cholesterol level was 4.56±1.53 mmol/L; triglycerides were 2.5±1.9 mmol/L; LDL-C was 2.94±1.21 mmol/L; and HDL-C was 0.49±0.21 mmol/L. The mean plasma lipid and lipoprotein cholesterol values for each group are shown in Table 1, and mutational analysis of the ABCA1 gene is shown in Table 2.
A 2D gel electrophoresis was performed on one normolipidemic control subject, one patient with TD, one patient with FHD, one patient with HypoA who had normal cellular lipid efflux, and one patient with HyperA (Figure 1). Identified in a normal control are pre-\(\beta\)/H\(9252\) lipoprotein AI (Lp-AI) and pre-\(\beta\)/H\(9252\) Lp-AI, as well as \(\alpha\)-Lp-AI particles. In the plasma of patients with TD and FHD, pre-\(\beta\) Lp-AI and pre-\(\beta\) Lp-AI particles were identified, but there is a marked reduction in \(\alpha\)-Lp-AI particles in FHD patients and a complete absence in TD. This suggests a failure of lipidation of nascent HDL particles in subjects with TD and FHD. The HypoA subject is characterized by decreased larger \(\alpha\)-Lp-AI particles (HDL\(2b\) and HDL\(2a\)). For comparison purposes, separation of apoAI-containing lipoprotein from a HyperA subject (HDL-C, 3.26 mmol/L) shows an increase in the mass and size of \(\alpha\)-Lp-AI, specifically the larger cholesteryl ester-rich particles (HDL\(2b\) and HDL\(2a\)).

Correlation Between Cellular Phospholipid and Cholesterol Efflux

Absolute normal control values for apoAI-mediated cellular phospholipid efflux at 24 hours ranged between 14% and 19%, and cholesterol efflux ranged between 13% and 16%. Mean efflux values for normal controls in each experiment were set at 100%, and efflux values in tested patients were then expressed as a percentage of controls. The results were then averaged for the number of experiments. The coefficient of variability for the efflux assays is \(\pm 5\%\). As shown in Figure 2, there is a close correlation between cellular cholesterol and phospholipid efflux \((r=0.72, P<0.001;\ \text{cholesterol efflux}=0.94\times\text{phospholipid efflux}+10)\).

Cellular Phospholipid and Cholesterol Efflux

The results of phospholipid (Figure 3A) and cholesterol (Figure 3B) efflux experiments are shown for normal and TD control subjects, newly diagnosed TD patients, FHD patients, undiagnosed HypoA subjects, and control HyperA subjects. The 2 TD control subjects have a markedly depressed phospholipid (19\(\pm\)2% and 16\(\pm\)6%) and cholesterol efflux (19\(\pm\)2% and 16\(\pm\)1%) values compared with normal control subjects. The FHD subjects had intermediate values between those of TD and normal control subjects, with a mean phospholipid efflux of 59\(\pm\)10% and cholesterol efflux of 56\(\pm\)15%. The remaining HypoA patients had normal phospholipid (99\(\pm\)25%) and cholesterol efflux (106\(\pm\)35%).

There was a wide biological variability with considerable overlap between HypoA subjects and normal control subjects. Conversely, patients with HyperA also had normal efflux values for phospholipid (94\(\pm\)18%) and cholesterol efflux (106\(\pm\)47%). For the present study, we defined an efflux defect as a cellular

### Table 2. Molecular Characterization of ABCA1 Gene in Study Subjects

<table>
<thead>
<tr>
<th>Cell Lines</th>
<th>HDL-C, mmol/L</th>
<th>Nucleotide Change</th>
<th>Predicted Protein Alteration</th>
</tr>
</thead>
<tbody>
<tr>
<td>TD CTL-1</td>
<td>0.10</td>
<td>Exon 30 T4369C; exon 24 splice site G-&gt;C</td>
<td>C1477R; part of the transcript deleted</td>
</tr>
<tr>
<td>TD CTL-2</td>
<td>0.15</td>
<td>Exon 13 A1730G</td>
<td></td>
</tr>
<tr>
<td>FHD-1</td>
<td>0.40</td>
<td>Exon 14 Δ2017–9</td>
<td>ΔL693</td>
</tr>
<tr>
<td>FHD-2</td>
<td>0.18</td>
<td>Exon 48 G6370T</td>
<td>R2144X</td>
</tr>
<tr>
<td>FHD-3</td>
<td>0.39</td>
<td>Exon 41 Δ5618–23</td>
<td>ΔDE1939.4</td>
</tr>
<tr>
<td>FHD-4</td>
<td>0.18</td>
<td>Exon 18 C2665T</td>
<td>R909X</td>
</tr>
<tr>
<td>FHD-5</td>
<td>0.10</td>
<td>Exon 23 T3667C</td>
<td>M1091T</td>
</tr>
<tr>
<td>FHD-6</td>
<td>0.57</td>
<td>Exon 49 C6844T</td>
<td>P2150L, R587W</td>
</tr>
<tr>
<td>TD-1</td>
<td>0.03</td>
<td>Exon 48 ΔG6370; ND</td>
<td>2145X</td>
</tr>
<tr>
<td>TD-2</td>
<td>0.07</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>TD-3</td>
<td>0.03</td>
<td>ND</td>
<td>2203X</td>
</tr>
<tr>
<td>TD-4</td>
<td>0.09</td>
<td>Exon 19 C3181T; ND</td>
<td>T929; ND</td>
</tr>
</tbody>
</table>

CTL indicates control. ND indicates not determined. Subjects FHD-1 to 6 are heterozygous for the reported mutations; TD-1,3 and TD CTL-2 are homozygous; and TD-4 and TD CTL-1 are compound heterozygous.

![Figure 1. 2D Gel electrophoresis separation of plasma apoAI-containing lipoproteins. Plasma from normolipidemic (Normal), Tangier disease (TD), familial HDL deficiency (FHD), HypoA with normal cellular lipid efflux (HypoA), and hyperalphalipoproteinemia (HyperA) subjects were separated according to charge (agarose, horizontal axis) and size (polyacrylamide gradient, 3% to 20% gel, vertical axis). ApoAI was detected with \(^{125}\)I-labeled anti-apoAI antibody. Molecular size markers are indicated on the left panel. ApoAI-containing HDL subpopulations are indicated with arrows.](http://circ.ahajournals.org/content/1368/15/1368/suppl/DC1/fig1.png)
phospholipid efflux $<70\%$ (the maximal phospholipid efflux value in known ABCA1 heterozygotes is $69\%$; Figure 3A). Using the linear regression analysis in Figure 2, this corresponds to a cellular cholesterol efflux value of $75\%$, and this is used as a cut-point for cholesterol efflux. In the HypoA subjects without known mutations of the ABCA1 gene, there was no significant correlation between HDL-C and plasma triglycerides or with total cholesterol, LDL-C, and non-HDL-C.

The prevalence of cellular lipid efflux defects and ABCA1 mutations was examined in the 64 HypoA subjects. There were 4 new TD and 6 FHD patients (10 of 64; 16\%). In addition, 7 additional patients had a phospholipid efflux defect (11\%) and 11 had a cellular cholesterol efflux defect (17\%). On the basis of this analysis, 17 of 64 (27\%) HypoA subjects have a cellular phospholipid efflux defect and 21 of the 64 (33\%) have a cholesterol efflux defect. Of these, 10 (4 TD and 6 FHD) have a known ABCA1 mutation (16\%). Interestingly, most of the patients with low HDL-C had a normal cellular phospholipid or cholesterol efflux, and $>40\%$ had values $>100\%$ of normal controls (Figure 3). Of the patients with HypoA and a cellular phospholipid efflux defect, family studies were performed. The ABCA1 gene locus was excluded in 2 families, whereas in 3 others, possible linkage with ABCA1 was found. This datum would suggest that the majority of efflux-defective HypoA subjects are due to mutations in the ABCA1 gene but also that other proteins might be involved in the cellular lipid efflux process.

As can be seen in Figure 4A, there was a significant relationship between cellular phospholipid efflux and HDL-C levels in patients with HypoA ($r=0.64$, $P<0.001$). A significant correlation was also observed between cholesterol efflux and HDL-C levels ($r=0.48$, $P<0.001$) (Figure 4B). However, this close relationship between cellular lipid efflux and HDL-C levels was attenuated once the HDL-C reached $\approx1.0$ mmol/L, suggesting that cellular lipid efflux is no longer a determinant of HDL-C levels beyond $1.0$ mmol/L (Figure 5).

**Discussion**

The present study shows that there is a marked correlation between cellular phospholipid and cholesterol efflux. In this analysis of 64 patients with low HDL-C, 27\% had a phospholipid efflux defect and 33\% had a cellular cholesterol efflux defect. Of those, 16\% (10 of 64) were identified as carriers of mutations within the ABCA1 gene, whereas 3 additional subjects showed a linkage with ABCA1 gene locus in their family. In 2 of these subjects (phospholipid and cholesterol efflux of $<70\%$ and $75\%$, respectively), family studies excluded the ABCA1 gene as a cause of the cellular lipid efflux defect, suggesting that other genes than ABCA1 might be involved in the cellular lipid efflux pathway. In the remaining subjects, the cause of the low HDL-C remains unknown.

Our results also showed (Figures 4 and 5) that cellular phospholipid efflux ($r=0.64$) is a better determinant of HDL-C levels than cholesterol efflux ($r=0.48$) in HypoA patients. This is consistent with the concept that ABCA1 acts primarily as a phospholipid translocase$^{21}$ and cholesterol efflux could occur subsequent to phospholipid efflux. However, in patients with HDL-C levels above $\approx1.0$ mmol/L, no
further relationship seems to exist between phospholipid efflux and plasma HDL-C levels (Figure 5). These data are in agreement with the concept that the early lipidation of lipid-poor apoAI is critical in the formation of nascent HDL particles but that other factors, including apoAI production rate, lecithin:cholesterol acyltransferase, cholesteryl ester transfer protein, and phospholipid transfer protein activities in plasma are further critical determinants of the maturation of the HDL particle and, therefore, of HDL-C levels.

We previously showed that a tight correlation exists between cellular cholesterol efflux and HDL-C level in our ABCA1 mutants. We extended this observation to show that this relationship exists regardless of mutation at the ABCA1 locus and that cellular phospholipid and cholesterol efflux are prime determinants of HDL-C levels for the early lipidation process. The 2-step hypothesis raised by Fielding et al is supported by the present data in that apoAI has to be first partially lipidated to become an acceptor of cellular cholesterol.

It is of interest to note that patients with HyperA (n=5) have normal mean values for cellular phospholipid and cholesterol efflux. This would suggest that the mechanism by which HDL-C is increased in these patients is not via the cellular lipid efflux pathway.

The prevalence of ABCA1 mutations in HypoA subjects observed in this study (16%) is supported by our previous study. The present study also shows that HDL deficiency is far more heterogeneous than previously thought. Thus, studies examining novel genes for HDL deficiency or quantitative trait loci must take into account that the HDL-C trait is genetically heterogeneous and that care in phenotyping must be taken when grouping families together for genome-wide scanning analysis.

The discovery of the ABCA1 transporter as a key regulator of cellular cholesterol efflux is supported by studies in humans showing that mutations within the gene cause profound HDL deficiency. Although the regulation of the ABCA1-mediated cellular lipid trafficking and efflux is complex and tissue-specific, initial lipidation of apoAI is rate limiting for the formation of cholesterol acceptor particles. Further lipidation with cholesteryl ester and triglycerides is coupled with the plasma enzyme lecithin:cholesterol acyltransferase and the transfer proteins cholesteryl ester transfer protein and phospholipid transfer protein, which enhance net transfer of cellular cholesterol and triglycerides onto an HDL particle; these events are ABCA1-independent.

Triglyceride-rich lipoproteins exchange their core lipids with HDL in vivo, a process that is facilitated by lipoprotein lipase and cholesteryl ester transfer protein. In the present study, we did not observe a correlation between plasma triglyceride and HDL-C levels in our HypoA subjects. Our data suggest that proteins other than ABCA1 may be involved in cellular lipid efflux onto apoAI and the formation of mature lipidated HDL particles in human plasma.

Further study of the role of peripheral cell ABCA1 in controlling plasma HDL-C levels may provide new insights into the mechanism of reverse cholesterol transport, plasma factors affecting HDL metabolism, and the therapeutic potential of ABCA1 in preventing or treating atherosclerotic vascular disease. In addition, the antitherogenic properties of HDL particles may not solely be related to their ability to

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Figure 4. Correlation between HDL-C and cellular phospholipid (A) and cholesterol (B) efflux. Data from 64 subjects with low HDL-C were plotted. Cholesterol or phospholipid efflux is expressed as percent of normal controls (which was set at 100%).

Figure 5. Relationship between plasma HDL-C and phospholipid efflux. A relative linear relationship between phospholipid efflux and HDL-C is observed at low levels of HDL-C. The correlation disappears when HDL-C exceeds 1.0 mmol/L. CTL indicates control.
remove cellular cholesterol but to modulate vascular endothelial function as well.⁶

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