The role of the ABCA1 transporter and cholesterol efflux in familial hypoalphalipoproteinemia

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Abstract Defects in the gene encoding for the ATP binding cassette (ABC) transporter A1 (ABCA1) were shown to be one of the genetic causes for familial hypoalphalipoproteinemia (FHA). We investigated the role of ABCA1-mediated cholesterol efflux in Dutch subjects suffering from FHA. Eighty-eight subjects (mean HDL cholesterol levels 0.63 ± 0.21 mmol/l) were enrolled. Fibroblasts were cultured and loaded with [^{3}H]cholesterol. ABCA1 and non-ABCA1-mediated efflux was studied by using apolipoprotein A-I (apoA-I), HDL, and methyl-β-cyclodextrin as acceptors. Efflux to apoA-I was decreased in four patients (4/88, 4.5%), and in all cases, a mutation in the ABCA1 gene was found. In the remaining 84 subjects, no correlation between efflux and apoA-I or HDL cholesterol was found. Efflux to both HDL and cyclodextrin, in contrast, did correlate with HDL cholesterol plasma levels ($r = 0.34$, $P = 0.01$; and $r = 0.27$, $P = 0.008$, respectively). The prevalence of defects in ABCA1-dependent cholesterol efflux in Dutch FHA patients is low. The significant correlation between plasma HDL cholesterol levels and methyl-β-cyclodextrin-mediated efflux in the FHA patients with normal ABCA1 function suggests that non-ABCA1-mediated efflux might also be important for plasma HDL cholesterol levels in these individuals.—Kees Hovingh, G., M. J. A. van Wijland, A. Brownlie, R. J. Bisoendial, M. R. Hayden, J. J. P. Kastelein, and A. K. Groen. **The role of the ABCA1 transporter and cholesterol efflux in familial hypoalphalipoproteinemia. *J. Lipid Res.* 2003.** 44: **1251–1255.**

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Decreased plasma levels of HDL cholesterol have consistently been shown to be associated with an increased risk for coronary artery disease (CAD). The atheroprotective role of HDL cholesterol is in part ascribed to its capacity to transport cholesterol from peripheral cells (including vascular macrophages) to the liver, a mechanism known as reverse cholesterol transport (RCT) (1).

A considerable number of proteins, enzymes, and receptors are involved in RCT, and in principle, all may play a role in the pathogenesis of atherosclerosis. Identification of novel genes and proteins in the pathway may be accomplished by family studies and genetic analysis. By means of this strategy, a crucial rate-controlling step in RCT has been identified by performing linkage analysis and mutation detection in families with familial hypoalphalipoproteinemia (FHA) not caused by LCAT or apolipoprotein A-I (apoA-I) mutations (2–4). The culprit gene was shown to encode a 250 kDa transmembrane protein called ATP binding cassette (ABC) transporter A1 (ABCA1). ABCA1 belongs to the family of ABC proteins and is expressed ubiquitously in human tissues (5). In peripheral cells, including vascular macrophages, ABCA1 regulates energy-dependent transport of cholesterol and phospholipids to apoA-I, the major protein in HDL. Mutations in the gene encoding ABCA1 give rise to decreased cholesterol and phospholipid efflux to apoA-I (6–8). Homozygosity for mutations in the ABCA1 gene causes Tangier Disease (TD), and heterozygosity is one of the causes for FHA. TD is characterized by near absence of plasma HDL cholesterol, whereas in ABCA1 heterozygotes, HDL cholesterol levels are approximately half the normal levels for sex and age. A strong correlation exists between cholesterol efflux capacity from fibroblasts and plasma HDL cholesterol levels in ABCA1 homo- and heterozygous patients (9).

The role of ABCA1 dysfunction in CAD risk has been the subject of debate. Before the molecular defect underlying TD was identified and the disease was diagnosed by clinical assessment, CAD was found to occur in less than half of cases over 35 years of age (10), which is a remarkably low incidence given the severity of HDL deficiency. It has been suggested that this relative protection from atherosclerosis in TD patients is caused by the low levels of atherogenic LDL cholesterol (11). Because of the small numbers of TD

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patients, the strong referral bias, and the prior inability to diagnose this disorder by genetic screening, however, it has been so far impossible to draw firm conclusions regarding the risk for CAD in TD patients. More recently, a 3-fold higher prevalence of symptomatic vascular disease was found in heterozygotes for ABCA1 (12). We have also described a strong correlation between cholesterol efflux and CAD risk, as measured by means of the surrogate marker, intima media thickness, in ABCA1 heterozygotes (9). In line with these findings, ABCA1 knockout mice were shown to suffer from enhanced atherosclerosis and, in contrast, overexpression of ABCA1 protected against diet-induced atherosclerosis (13, 14). These findings further confirmed our observations in humans that ABCA1 dysfunction does indeed promote atherogenesis.

An important unanswered question is how often dysfunctional ABCA1-mediated efflux underlies FHA. Mott and colleagues reported that ABCA1-mediated cholesterol efflux was reduced in 50% of the 14 probands tested. An ABCA1 mutation was found in four of those cases, suggesting that 29% (4/14) of these Canadian patients suffered from FHA due to ABCA1 mutations (15).

The aim of the present study was to establish the prevalence of ABCA1-mediated cholesterol efflux defects in a cohort of Dutch patients with familial low HDL. We therefore measured in the fibroblasts of our large FHA cohort the cholesterol efflux to apoA-I, reflecting ABCA1-mediated efflux, and to cyclodextrin and HDL, reflecting non-ABCA1-mediated efflux. DNA from all patients with defects in ABCA1-mediated efflux was subjected to sequencing of the ABCA1 gene.

METHODS

Patients

Patients were recruited from the Vascular Research Network in The Netherlands and referred to the Academic Medical Center, Amsterdam. The main inclusion criterion was that the patient should have an isolated low HDL cholesterol level (below the fifth percentile, corrected for age and sex). FHA was defined as the presence of a similar lipid abnormality in at least one first-degree family member. Informed consent was obtained from all subjects for plasma sampling, storage, and genetic analysis, under a protocol approved by the Ethics Committee of the Academic Medical Center in Amsterdam.

Lipids and lipoproteins

Blood samples were collected in EDTA-containing tubes from all participants following an overnight fast. Plasma total cholesterol was measured with an enzymatic colorimetric procedure (CHOD-PAP, Boehringer Mannheim, Mannheim, Germany) as previously described (16). HDL cholesterol was determined after precipitation of apoB-containing lipoproteins with phosphotungstic acid and magnesium. LDL cholesterol was calculated using the Friedewald formula (17). Triglycerides were measured using an enzymatic colorimetric method using lipase, glycerol kinase, and glycerol-3-phosphate oxidase.

Efflux studies

Fibroblasts were obtained from participants by explant culture from a 3 mm punch biopsy at a 1 mm skin thickness and were cultured (until passages 5–15) in 24-well culture plates until full confluency, essentially as described earlier (9). The culture medium was DMEM supplemented with 10% fetal calf serum. After washing with DMEM, cells were loaded with 30 μg/ml [^{3}H]cholesterol (38 Ci/mmol) in DMEM over 24 h. After cells were washed five times with PBS-BSA 0.2% (w/v), the efflux assay was started by adding 5 μg/ml apoA-I, 50 μg/ml HDL_3, or 1 mM methyl-β-cyclodextrin to the wells. After 20 h incubation at 37°C, the medium was collected and centrifuged. Subsequently, [^{3}H]cholesterol was quantified by liquid scintillation counting. Total cellular [^{3}H]cholesterol was determined after extraction of the cells with 2-propanol. The percentage efflux was calculated by dividing the radioactive counts in the efflux medium by the sum of the counts in the medium and the cell extract. Efflux to apoA-I and HDL was linear over the time span of the experiment (unpublished observations).

Normal values for efflux to the different acceptors were determined in fibroblasts derived from a group of 11 healthy normolipidemic volunteers. Plasma HDL cholesterol levels in those subjects were shown to be above the 15th percentile, corrected for age and sex.

To test the effect of the passage number on cholesterol efflux, fibroblasts of five controls were cultured to passages 9 to 20 and cholesterol efflux was measured. No significant effect of passage number on efflux was discerned.

Mean efflux to apoA-I in this control group was 9.86% of total cholesterol in 20 h. (SD 2.71%; 95% confidence interval, range 6.49% to 14.31%). Efflux to apoA-I was considered to be abnormal at a value below 4.45% (mean efflux in controls minus 2 SD).

Mutation detection and sequence analysis

Leucocytes were isolated from buffy coat for DNA extraction. For mutation detection, all forward and reverse PCR primers, flanking each exon, were designed with Repeat Master (http://ftp.genome.washington.edu/cgi-bin/Repeat Master) and Primer3 (http://www-genome.wi-mit.edu/genome_software/other/primer3.html). The PCR protocol has been previously described (16).

Statistical analysis

Lipid values are displayed as mean ± SD. Student's *t*-test for unpaired data was used to compare individuals with ABCA1 defects with those not having defective apoA-I-mediated efflux. A linear regression model was used for the correlation analysis between plasma HDL cholesterol level and cholesterol efflux to the different acceptors. The Hotelling *t*-test was used to test the difference in correlations between HDL cholesterol plasma level and HDL-apoA-I efflux and cyclodextrin efflux. All data were statistically analyzed using SPSS software.

RESULTS

Over the course of three years, 106 patients with HDL cholesterol levels below the 5th percentile for sex and age were identified. Eighteen patients were excluded from further study based on the absence of low HDL in a first-degree family member. Characteristics of the remaining 88 patients enrolled in this study are summarized in **Table 1**. A skin biopsy was performed in all participants, and fibroblasts were cultured. After growth to confluency, the cholesterol efflux to apoA-I, rHDL, or the aspecific acceptor methyl-β-cyclodextrin was measured. Efflux to apoA-I was found to be significantly decreased in only four patients (4.5%). Genetic analysis revealed that these patients were either compound heterozygotes or heterozygotes for

TABLE 1. Baseline characteristics of 88 individuals with FHA

Demographics	
M/F	75/13
CAD/PVD, n (percentage of total)	51 (58)
Age, years, mean ± SD	53.09 ± 11.81
Lipids and lipoproteins	*mmol/l, mean ± SD*
TC	4.28 ± 1.08
LDL cholesterol	2.77 ± 1.07
HDL cholesterol	0.63 ± 0.21
TG	1.83 ± 1.07

CAD, coronary artery disease; FHA, familial hypoalphalipoproteinemia; M/F, male-female ratio; PVD, peripheral vascular disease; TC, total cholesterol; TG, triglycerides.

mutations in the gene encoding for ABCA1. The two compound heterozygous patients have been described previously [one of the compound heterozygous carriers suffered from a missense mutation (T to C at position 4,369) resulting in a C1477R, and a defect (IVS24 + 1G to C) that caused differential splicing, whereas the other was shown to carry a missense mutation (C to T at position 3,181 resulting in T929I) and a de novo nonsense mutation] (16). A missense mutation (T3212C) resulting in M1091T, and a C to T nucleotide substitution at position 6,844 resulting in P2150L were the defects in the two heterozygous carriers. Thirteen patients had very low levels of plasma HDL cholesterol (<0.5 mmol/l) and normal cholesterol efflux to apoA-I, indicating that non-ABCA1-related factors must be responsible for such isolated low HDL in these subjects. In six of these 13 patients, we discovered a novel mutation in the gene encoding apoA-I (mutation L178P; unpublished observations). In addition, another one of these 13 patients had two defects in the LCAT gene (unpublished observations). Efflux to apoA-I of all remaining 84 patients was in the normal range (4.45–14.3%).

To assess the relationship between efflux to other cholesterol acceptors and plasma HDL cholesterol levels, efflux measurements to various acceptors were plotted against HDL cholesterol levels. **Figure 1** shows the correlation between apoA-I efflux and plasma HDL cholesterol levels in the entire study population. Interestingly, no correlation between the two parameters is evident. However, efflux to HDL was significantly ($r = 0.34$, $P = 0.001$) correlated with serum HDL cholesterol levels (**Fig. 2**). This correlation may be partially due to the presence of patients with ABCA1 mutations. Nevertheless, when this analysis was repeated excluding these four patients, the correlation between HDL cholesterol and efflux to HDL remained significant ($r = 0.26$, $P = 0.05$).

By subtracting apoA-I-mediated efflux from HDL-mediated efflux, the influence of ABCA1-independent processes may be evaluated. As depicted in **Fig. 3**, this residual efflux also correlated significantly with plasma HDL cholesterol in our patients ($r = 0.32$, $P = 0.008$). In contrast, there was no correlation between efflux to HDL and plasma levels of total cholesterol or triglycerides. Efflux to methyl-β-cyclodextrin, an independent method to monitor nonspecific cholesterol efflux, and plasma levels of HDL cholesterol were

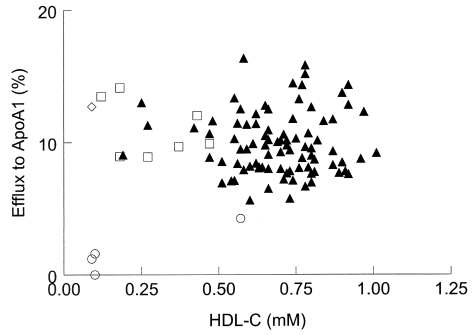

Fig. 1. Lack of correlation between efflux of cholesterol to apolipoprotein A-I (apoA-I) and plasma HDL cholesterol. Fibroblasts derived from 88 patients with familial hypoalphalipoproteinemia (FHA) were loaded with [^{3}H]cholesterol as described in Methods, and efflux to 5 μg/ml apoA-I was monitored. Data are expressed as percentage of total cellular cholesterol effluxed to apoA-I during a 20 h incubation. Closed triangles, FHA patients; open circles, patients with ATP binding cassette (ABC) transporter A1 (ABCA1) mutations; open squares, patients with apoA-I mutations; and open diamond, patient with LCAT mutation.

significantly correlated ($r = 0.27$, $P = 0.008$). The correlation values for HDL minus apoA-I efflux and cyclodextrin efflux did not differ significantly (Hotelling *t*-test).

DISCUSSION

In our study, we determined the prevalence of defects in ABCA1-mediated cellular cholesterol efflux in a large cohort of Dutch patients with isolated low HDL cholesterol. We show that such defects account for a small percentage (4.5%) of low HDL cholesterol patients. Mutations in ABCA1 were found in all cases that exhibited impaired ABCA1-mediated efflux. This finding is in sharp contrast to the results of Mott et al., who found impaired cholesterol efflux (to apoA-I) from fibroblasts in 50% of the included 14 hypoalphalipoproteinemic patients. Fur-

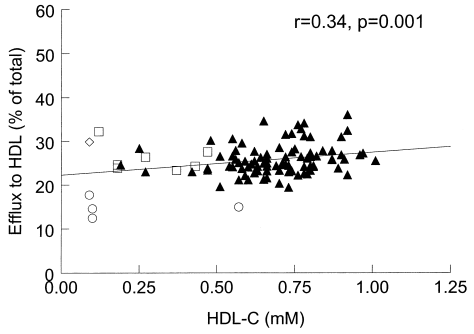

Fig. 2. Cholesterol efflux to HDL correlates with plasma HDL cholesterol. Fibroblasts derived from 88 patients with FHA were loaded with [^{3}H]cholesterol as described in Methods, and efflux to 50 μg/ml HDL was determined. Data are expressed as percentage of total cellular cholesterol effluxed to HDL during a 20 h incubation. Closed triangles, FHA patients; open circles, patients with ABCA1 mutations; open squares, patients with apoA-I mutations; and open diamond, patient with LCAT mutation.

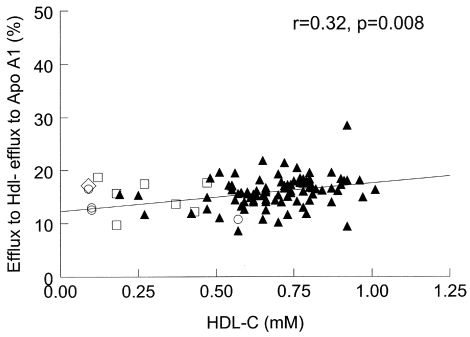

Fig. 3. Relation between the apoA-I-independent cholesterol efflux to HDL and plasma HDL cholesterol concentration. Values for apoA-I-independent cholesterol efflux to HDL were calculated by subtracting the values for efflux to apoA-I given in Fig. 1 from the values for efflux to HDL given in Fig. 2. Closed triangles, FHA patients; open circles, patients with ABCA1 mutations; open squares, patients with apoA-I mutations; diamond, patient with LCAT mutation.

ther genetic analysis revealed that 29% (4/14) of those patients were carriers of an ABCA1 mutation. On the basis of these data, the authors suggested that a defect in cellular cholesterol efflux is a common cause of moderate to severe hypoalphalipoproteinemia (15).

The difference in prevalence of defects in ABCA1-mediated efflux and ABCA1 mutations in our low HDL cholesterol patients could be a consequence of selection bias. Whereas Mott et al. recruited their patients from the French Canadian population, we retrieved our patients from the general Dutch population. It has been well established that the prevalence of genetic variation depends on the selected population, and in this case, the selection per se might be the cause for the observed differences. Moreover, Mott and colleagues considered a >40% reduction in ABCA1-mediated efflux as abnormal. We defined the lower limit as the mean efflux in 11 healthy, normolipidemic controls minus 2 × SD. Our lower limit equals an almost 50% reduction of the average of the 11 healthy controls (4.45/9.86 × 100 = 45%). Application of the 40% reduction limit of Mott et al., which would result in an absolute apoA-I value in our study of 5.9%, would only increase by one the number of patients to be sequenced and hence does not account for the much lower prevalence of efflux defects in our population. On the basis of our results, we might conclude that defects in ABCA1 are an infrequent cause for FHA in The Netherlands.

Our study does not allow an estimate of the true prevalence of ABCA1 mutations in the Dutch population. In a recent study, Wellington et al. showed that cholesterol efflux in patients with one defective ABCA1 allele decreases significantly only when the mutation leads to a truncated protein (18). Apparently the presence of a truncated ABCA1 molecule negatively impacts on the activity of the intact protein. Thus, we might underestimate the prevalence of ABCA1 mutations in the general population, insofar as some amino acid substitutions may only be associated with a very mild phenotype. Similar conclusions about the role of ABCA1 in FHA were recently described in a study focusing on the genetics rather than on the activity of ABCA1 (19).

As we have previously reported, in the subset of patients with ABCA1 mutations, we observed a strong correlation between efflux to apoA-I and plasma HDL levels (9), indicating an important role for ABCA1 in these patients. However, this correlation could not be generalized to the remainder of the cohort, in which efflux to apoA-I showed no correlation to plasma HDL levels. Interestingly, efflux of cholesterol to HDL particles showed significant correlation to plasma HDL. We hypothesize that efflux of cholesterol to lipid-poor apoA-I is primarily controlled by ABCA1, whereas efflux to more mature HDL depends on other factors as well. In line with this is the observation that fibroblasts or macrophages derived from patients with Tangier Disease show negligible efflux toward apoA-I, but efflux to HDL is reduced by only 50% in these patients (7, 8). The nature of this non-ABCA1-dependent cholesterol efflux is not well understood. It may be due to passive diffusion of cholesterol, but unidentified proteins are likely to be involved in this process. Low HDL cholesterol levels were found to be hereditary, but our study cannot address the question of whether the non-ABCA1-dependent efflux to HDL was inherited as well.

Assuming that the apoA-I-dependent component in efflux to HDL is saturated, the apoA-I-independent component may be calculated by subtracting the apoA-I-mediated efflux from the HDL-mediated efflux (Fig. 3). An alternative and perhaps better way to determine apoA-I-independent efflux is to use methyl-β-cyclodextrin as an acceptor. In line with other studies (20, 21), this compound induced a high rate of cholesterol efflux in an apparently aspecific way. Interestingly, also for this parameter, a significant correlation between plasma HDL cholesterol and efflux was observed (**Fig. 4**), which might suggest that nonspecific, apoA-I-independent efflux to mature HDL particles might contribute importantly to circulating HDL cholesterol levels.

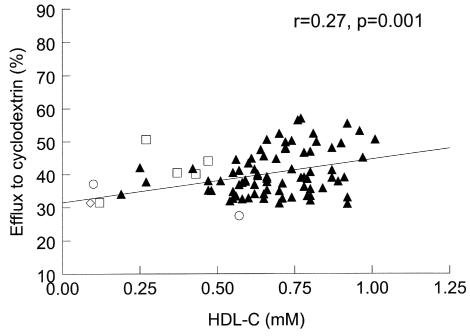

Fig. 4. Aspecific cholesterol efflux to methyl-β-cyclodextrin correlates with plasma HDL cholesterol concentration. Fibroblasts derived from 88 patients with FHA were loaded with [^{3}H]cholesterol as described in Methods, and efflux to 1 mM methyl-β-cyclodextrin was measured. Data are expressed as percentage of total cellular cholesterol effluxed to methyl-β-cyclodextrin during a 20 h incubation. Closed triangles, FHA patients; open circles, patients with ABCA1 mutations; open squares, patients with apoA-I mutations; diamond, patient with LCAT mutation.

Our data therefore suggest that non-ABCA1-mediated cholesterol efflux is important for HDL cholesterol levels in this group of patients and that other, yet to be discovered genetic defects might be responsible for the low levels of HDL cholesterol found in these patients. Further research is required to identify these novel genes and to elucidate possible novel pathways involved in HDL metabolism.

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