Infusion of Reconstituted High-Density Lipoprotein Leads to Acute Changes in Human Atherosclerotic Plaque

James A. Shaw, Alex Bobik, Andrew Murphy, Peter Kanellakis, Peter Blombery, Nigora Mukhamedova, Kevin Woollard, Stuart Lyon, Dmitri Sviridov, Anthony M. Dart

Abstract—Studies have shown a reduction in plaque volume and change in plaque ultrasound characteristics after 4 infusions of reconstituted high-density lipoprotein (rHDL). Whether rHDL infusion leads to acute changes in plaque characteristics in humans is not known. Patients with claudication scheduled for percutaneous superficial femoral artery revascularization were randomized to receive 1 intravenous infusion of either placebo or rHDL (80 mg/kg given over 4 hours). Five to 7 days following the infusion, patients returned and revascularization was performed including atherec- tomy to excise plaque from the superficial femoral artery. Twenty patients (17 males) average age, 68 ± 10 years (mean ± SD) were recruited. Eleven patients had a history of documented coronary artery disease, all patients were on aspirin, and 18 were on statins. Ten of the patients received rHDL and 10 placebo. There was significantly less vascular cell adhesion molecule-1 expression (28 ± 3% versus 50 ± 3%; P < 0.05) and a reduction in lipid content in the plaque of HDL-treated subjects compared to placebo. The level of HDL cholesterol increased by 20% after infusion of rHDL and the capacity of apolipoprotein B–depleted plasma to support cholesterol efflux increased. Intravenous infusion of a single dose of reconstituted HDL led to acute changes in plaque characteristics with a reduction in lipid content, macrophage size, and measures of inflammation. These changes may contribute to the cardioprotective effects of HDL. (Circ Res. 2008;103:1084-1091.)

Key Words: lipoproteins ■ atherosclerosis ■ inflammation

Atherosclerotic disease remains the leading cause of death in the developed world. However, there has been a significant reduction in the morbidity and mortality from ischemic heart disease over the last 2 decades, largely as a result of better risk factor management. The introduction of statins, the competitive inhibitors of hydroxymethylglutaryl-coenzyme A reductase, has played a major role in reducing events in patients with documented atherosclerotic disease.1,2 The benefit provided by these drugs results predominantly from their ability to safely and effectively lower low-density lipoprotein (LDL).

Despite these advances, the majority of vascular events are not prevented. Clearly, other risk factors need to be identified and addressed to help further reduce the burden of atherosclerosis. Epidemiological studies have shown a strong inverse correlation between circulating high-density lipoprotein (HDL) cholesterol and the risk of ischemic heart disease.3,4 Low HDL levels have also been shown to be a predictor for the development of symptomatic peripheral vascular disease.5,6 In recent years, both oral and intravenous agents resulting in an increase in serum HDL levels have become available.7 Small human studies using different forms of intravenous HDL infusions have shown varying results. Two studies have shown that the amount of coronary plaque, as measured with intravascular ultrasound (IVUS), was significantly reduced in the HDL-treated group.8 The ERASE study did not show that the change in plaque volume differed between the HDL and placebo groups;9 however, differences in plaque characteristics were observed in the HDL group.9

The presumed cardioprotective effect of HDL is likely to be multifactorial and include not only its role in reverse cholesterol transport but also antiinflammatory, antioxidant, and antithrombotic effects.10,11 Animal studies have shown that increases in HDL acutely change plaque composition.12-14

Although there have been studies showing that plaque characteristics, as assessed with IVUS, change in the coronary circulation after HDL infusions, histological changes have not been studied. The primary goal of this study was to determine the effects of reconstituted HDL (rHDL) on serum markers of inflammation and on atherosclerotic plaque composition. This may improve our understanding of how HDL provides benefit in patients with cardiovascular disease.
disease and provide support for the use of such therapies in patients with coronary artery disease. We have studied patients with peripheral vascular disease because the plaque can be excised safely percutaneously and previous pathological studies have shown that plaque composition is similar in the different vascular beds.15

Materials and Methods
All patients gave their informed consent to the study, which was approved by the Human Ethics Committee of the Alfred Hospital and conducted in accordance with the principles of the Declaration of Helsinki 2000.

Patients
Patients were recruited if they had symptom limiting claudication despite medical management, including an exercise program, and had a lesion in their superficial femoral artery deemed suitable for percutaneous revascularization. We excluded patients <40 years of age and those with other major comorbidities, including cancer, with an expected survival of <12 months. Enrolled patients continued all of their usual medications, except for statins, which were discontinued 1 week before the infusion and were not recommenced until after the atherectomy/angioplasty. Statins were ceased before the infusion because it is known that statins elevate HDL levels. Because all patients clearly had vascular disease and more than half had documented coronary artery disease, it would be unethical to withhold statins for a prolonged period of time. We felt that ceasing statins 1 week before the infusion (of either placebo or rHDL) would limit any impact in confounding the effects of the HDL infusion without presenting ethical problems.

On the day of the infusion, patients came to the hospital in the morning having fasted from midnight the previous night. On arrival, venous access was obtained, and 30 mL of blood was withdrawn into EDTA tubes spun and stored for later analysis, again looking at lipid levels and inflammatory markers. Measurement of ankle brachial index was performed in the standard manner.

The infusion of either rHDL or placebo (saline solution) was given in the human research laboratory of the Department of Cardiology, Alfred Hospital. Patients were randomly assigned to 1 of the 2 therapies. The infusions were given intravenously over a 4-hour period in a double-blinded fashion, and the rHDL was given at a dose of 80 mg/kg body weight. rHDL (CSL Behring AG, Bern, Switzerland; product CSL 111) was reconstituted with 50 mL of sterile water for injection yielding a molecular weight: 775 Da.

Plasma Analysis
Plasma was stored at −70°C. Lipid levels, including total cholesterol, HDL cholesterol, and triglycerides, as well as liver function tests, were analyzed using an Abbott Architect ci 8200 blood analyzer by colorimetric assays. LDL was calculated using the Friedewald formula. Highly sensitive C-reactive protein was measured using an Abbott Architect ci 8200 with reagents from Roche Australia.

Soluble (s)P-selectin, soluble intercellular adhesion molecule (sICAM-1), and tumor necrosis factor (TNF)-α levels were measured by ELISA in the plasma of subjects both pre- and postinfusion (R&D). Plasma levels of soluble ICAM-1 rather than soluble vascular cell adhesion molecule (VCAM)-1 were examined because, despite increased VCAM-1 expression in human atherosclerotic lesions,17 plasma levels of soluble VCAM-1 are not increased in subjects with atherosclerosis;18,19 rather, plasma soluble ICAM-1 is strongly associated with atherosclerosis.18,19

Monocyte Activation
CD11b expression on peripheral blood monocytes was measured as a marker of monocyte activation. Peripheral blood anticoagulated with citrate was incubated with an anti-human CD11b antibody (Serotec, Clone ICRF44) 1:40 dilution for 15 minutes at 37°C. Samples were then fixed and red blood cells lysed with OptiLyse B (Immuneon) for 10 minutes with occasional mixing. The lysis process was completed with the addition of distilled water. Samples were controlled for by using the isotype matched negative control (FITC-antimouse IgG, Serotec, Clone W3/25). CD11b expression was measured by flow cytometry using FACs Calibur (Becton Dickinson). Analysis was conducted using the Cell Quest Pro software.

Cholesterol Efflux
To test the ability of HDL to support cholesterol efflux, apoB-containing lipoproteins were removed by precipitation with heparin/ manganese chloride (1.3 mg/mL and 0.092 mol/L, respectively), leaving HDL as the sole lipoprotein in incubation plasmas. Cholesterol efflux was measured after incubating apoB-depleted plasmas with human THP-1 cells activated by 48 hours of incubation with LXR agonists TO-901317 (4 µmol/L, Sigma) in which the cholesterol had been prelabeled over 48 hours with [1H]cholesterol (Amersham; specific radioactivity, 1.8 TBq/mmol; final radioactivity, 75 kBq/mL). After labeling, cells were washed, incubated for 18 hours in serum-free medium in the presence of the TO-901317 (final concentration, 4 µmol/L), and then incubated for a further 2 hours at 37°C with serum-free medium containing apoB-depleted plasma from the subjects at a final concentration of 2%. Radioactivity in media was assayed in a β counter. Cells were scraped from the plates and dissolved in 0.5 mol/L NaOH, and aliquots were assayed for protein content and radioactivity. Cholesterol efflux was determined as the radioactivity in medium divided by that in medium plus cells, after subtracting background efflux in control incubations without added acceptor (usually 10% of total) and expressed as a percentage. Each assay was performed in triplicate (coefficient of variation, <3%).

Plaque Analysis
Excised specimens were cut into 3 or more segments, depending on their size and embedded in OCT compound (Tissue-Tek), frozen in isopentane liquid nitrogen, and stored at −80°C until use. For the histological studies, 6-µm serial sections were cut from each of 3 blocks from each patient. Sections were stained with oil red O for lipid content.20 Immunohistochemistry was performed as previously described.21 Briefly, the sections were fixed in cold (−20°C) acetone.
Table 1. Clinical Characteristics of Patients and Medication Use in the Two Groups

<table>
<thead>
<tr>
<th></th>
<th>rHDL (n=10)</th>
<th>Placebo (n=10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, yr</td>
<td>71±8</td>
<td>65±12</td>
</tr>
<tr>
<td>Diabetes, n</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>Hypertension, n</td>
<td>6</td>
<td>4</td>
</tr>
<tr>
<td>Smoker, n</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>History of CAD, n</td>
<td>6</td>
<td>5</td>
</tr>
<tr>
<td>Ankle brachial index</td>
<td>0.6±0.05</td>
<td>0.7±0.07</td>
</tr>
<tr>
<td>Aspirin, n</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Statins, n</td>
<td>9</td>
<td>9</td>
</tr>
<tr>
<td>ACE inhibitors, n</td>
<td>7</td>
<td>7</td>
</tr>
</tbody>
</table>

CAD indicates coronary artery disease; ACE indicates angiotensin-converting enzyme.

Table 2. Lipid and CRP Measurements Pre- and Postinfusion in the Two Groups

<table>
<thead>
<tr>
<th></th>
<th>rHDL</th>
<th>PLACEBO</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total cholesterol, mmol/L</td>
<td>5.4±0.3</td>
<td>6.3±0.3*</td>
</tr>
<tr>
<td>LDL, mmol/L</td>
<td>3.5±0.2</td>
<td>4.0±0.2*</td>
</tr>
<tr>
<td>HDL, mmol/L</td>
<td>1.1±0.1</td>
<td>1.3±0.1*</td>
</tr>
<tr>
<td>Triglycerides, mmol/L</td>
<td>1.9±0.2</td>
<td>2.2±0.2*</td>
</tr>
<tr>
<td>HS CRP, mg/L</td>
<td>2.9±0.9</td>
<td>2.4±0.8</td>
</tr>
</tbody>
</table>

Data are means±SEM. *P<0.05 vs preinfusion in rHDL group; †P<0.05 vs preinfusion in placebo group. HS CRP indicates highly sensitive C-reactive protein.

Results

Baseline Characteristics
There was a total of 20 patients in the study, of whom 10 received rHDL and 10 placebo. Baseline characteristics and medication use are shown in Table 1. The two groups were matched for age: 71±8 years (mean±SD) in the rHDL group versus 65±11 years in the placebo group and risk factor profiles. Six patients in the rHDL group and 5 in the placebo group had a history of angiographically proven coronary artery disease (lesion of ≥50% in at least one major epicardial artery). Medication use was also similar between the groups with regard to the use of aspirin, statins, and angiotensin-converting enzyme inhibitors (Table 1).

All lesions were in the superficial femoral artery, 5 in the proximal, 6 the mid-, and 5 the distal superficial femoral artery (SFA), whereas 4 of the lesions involved the proximal and mid-SFA. Eleven of the lesions were total occlusions, and all lesions underwent atherectomy with successful excision of plaque.

Lipid Levels
Total cholesterol and LDL cholesterol were similar in the groups before treatment (5.9±0.3 versus 5.4±0.3 mmol/L and 3.7±0.3 versus 3.5±0.2 mmol/L).

In patients treated with rHDL, there was a significant increase in plasma concentrations of total cholesterol, LDL cholesterol, HDL cholesterol, and triglycerides following the infusion (Table 2). In the placebo group, the only difference seen post infusion was a decrease in the HDL (1.2±0.1 [pre] versus 1.1±0.1 mmol/L [post]; P<0.05).

Liver Function Tests
Liver function tests were performed before and after infusion in both groups. There were no significant changes in either group postinfusion (data not shown).

Inflammatory Markers
Highly sensitive C-reactive protein was similar in the 2 groups at baseline, and there was no difference following the infusion in either the placebo or rHDL-treated groups (Table 2).

In the other inflammatory plasma markers, there was a strong trend toward a decrease in TNF-α after rHDL infusion (P=0.05), whereas monocyte activation, as determined by CD11b expression, decreased following rHDL infusion (P<0.05). There was a trend toward a reduction in sP-selectin (P=0.08) but no change in circulating sICAM-1 levels between treatments (Figure 1).

Cholesterol Efflux
Figure 2 shows cholesterol efflux to plasma depleted of apoB-containing lipoprotein in absolute values (Figure 2A and 2C) and normalized to the preinfusion values (Figure 2B and 2D). After infusion, there was a 15% increase in the capacity of plasma to support cholesterol efflux from activated human macrophages in the HDL group (Figure 2A and 2B; P<0.005), whereas in the placebo group, there was no change (Figure 2C and 2D). When changes in cholesterol efflux were normalized to changes in the HDL concentration in the HDL group, the difference in the efflux to pre- versus postinfusion plasma disappeared. Moreover, there was a...
strong correlation between HDL-C level and cholesterol efflux ($r=0.54$, $P<0.002$). Thus, the major cause of increased capacity of plasma to facilitate cholesterol efflux after rHDL infusion is rise in HDL concentration, with changes in HDL functionality playing, if any, a minor role.

Size distribution of HDL in plasma was analyzed in both groups using nondenaturing gradient polyacrylamide gel electrophoresis, as described previously. No difference in HDL size distribution was found between the groups or between pre- and postinfusion plasma samples (data not shown).

**Plaque Changes**

Significant differences were seen in the plaque of the patients who received rHDL compared to placebo. Lipid in the plaque was assessed using oil red O staining, and there was less lipid
in the plaque treated with rHDL compared with those that received placebo. Figure 3 shows typical photomicrographs of the oil red O staining in the 2 groups. The average oil red O staining area per field was 49 537±4833 μm² in placebo group compared with 18 762±6302 μm² in the HDL group (P<0.05). Because macrophages increase in size on accumulating lipids,25 macrophage size was also compared between the 2 groups. The average cell size was significantly higher in the placebo group (832±367 μm²) compared to the rHDL group (349±103 μm²; P<0.05) (Figure 3). We also determined the effect of rHDL on the expression VCAM-1 in lesions. VCAM-1 was highly expressed in lesions and in cell-rich regions of plaque containing smooth muscle cells and macrophages and appeared associated with these cells, as previously shown.17 VCAM-1 expression was lower in the treated plaques compared with the placebo group (Figure 4). The percentage of cells expressing VCAM-1 was 28±3% in the rHDL group compared with 50±3% in the placebo group (P<0.05).

Discussion
We believe that this is the first in vivo human study to show that treatment with rHDL can induce acute changes in human atherosclerotic plaque composition. Following a single infusion of rHDL, there was significantly less lipid in the plaque and less inflammation (as measured by the presence of CD68 antigen) compared to the placebo group (Figure 4). This suggests that rHDL may be a promising therapeutic agent for the treatment of atherosclerotic plaques.
VCAM-1 positive cells), and macrophages in the plaque were also smaller. The significant reduction in neutral lipids associated with the smaller macrophage size seen in the plaque of rHDL-treated subjects was most probably the result of increased reverse cholesterol transport in these patients in response to the rHDL. Similar effects on plaque characteristics have been seen in apoE-deficient mice following a single infusion of apoA-Imin or with a reduction in plaque lipid and macrophage content.12 Both in the study of Shah et al, where the changes occurred within 48 hours of HDL being elevated, and in the present study, where the changes were seen 5 to 7 days after the infusion, rHDL acutely reduces plaque lipid levels. Furthermore, studies have shown that increasing HDL in apoE-deficient mice changes the lesion phenotype to a phenotype that is more stable by promoting migration of macrophages out of the plaque and increasing smooth muscle cell content.26 This could also help explain the reduced lipid seen in the plaques after rHDL treatment.

We also assessed the capacity of plasma to support cholesterol efflux from cultured cells and found this to be increased in the rHDL-treated patients. Thus, we have shown that the infusion of rHDL leads to increased cholesterol efflux in vitro and to a reduced concentration of cholesterol in macrophages in plaque. The role of HDL in increasing reverse cholesterol transport is likely to be a major contributor to its role in reducing cardiovascular events.27 In the present study, using rHDL, we did find evidence of increased reverse cholesterol transport: the capacity of plasma to support cholesterol efflux from human monocytes activated with LXR agonist (to increase contribution of specific, ATP binding cassette transporters-dependent efflux) after rHDL infusion. This increase was mainly attributable to higher levels of HDL because neither distribution of HDL between subfractions nor HDL functionality was significantly affected.

HDL is known to have other beneficial effects beyond reverse cholesterol transport. These include antiinflammatory28 and antithrombotic effects,10 as well improved endothelial function.29,30 The reduction in VCAM-1–positive cells seen in the plaque of the patients treated with rHDL suggests a reduction in the level of inflammation in these patients. The role of inflammation in both the development of atherosclerosis and its role in plaque disruption have been well studied. Increased inflammation is a feature of ruptured plaques.31 In the serum of the rHDL-treated patients, we found no change in highly sensitive C-reactive protein, but we did find a reduction in TNF-α and in monocyte activation, as assessed by CD11b activity. These changes suggest that rHDL reduces both systemic and plaque inflammation, a potentially important cardioprotective effect.

In the ERASE study using the same formulation used in the present study, 4 infusions of either rHDL or placebo were given to patients with acute coronary syndromes. In the rHDL group, there was a significant reduction in the plaque volume compared to baseline, as measured by IVUS; however, there was no significant difference between the treated and placebo groups. Furthermore, in the rHDL group, changes were seen in the IVUS studies, suggesting changes in plaque histology.9 In the present study, we have shown histologically that, in the peripheral vasculature rather than the coronary arteries, HDL
does acutely lead to changes in plaque histology, as has been suggested from the IVUS studies. The other major clinical trial using an intravenous form of HDL used apoA-I [5], which is a recombinant human apoA-I formulated in a complex with a naturally occurring phospholipid to mimic the properties of nascent HDL. In that study, 5 weekly infusions were given of either apoA-I [5] or placebo, with changes in plaque volume assessed by IVUS. There was a significant \underline{reduction in plaque volume in the apoA-I group. Although this absolute reduction was greater than in the ERASE study, this can be partly explained by the higher baseline plaque volume present in the treated group compared to the placebo group, which was not the case in the ERASE study. Plaque characteristics were not analyzed in the study using apoA-I [5].}

The dose used in the present study was 80 mg/kg, similar to the early human studies\(^{29,30}\) and at the start of the ERASE study, before it was reduced to 40 mg/kg because of concerns of liver toxicity. Whereas abnormalities in liver function were seen in those patients receiving 80 mg/kg and 40 mg/kg in the ERASE study, we found no abnormalities in our patients. However, we assessed liver function 5 to 7 days following the infusion, on the day of the atherectomy procedure, whereas in the ERASE study, the abnormalities seen in liver function peaked the day following the drug administration and declined over the subsequent days.

Changes in circulating HDL concentrations following rHDL infusion are related to the time interval between such infusions and blood sampling. Thus, in 2 previous studies using identical infusion protocols to the present study, plasma HDL concentration was increased by up to 100\% when assessed at 3 hours following infusion.\(^{29,30}\) In the present study, small but significant increases in HDL were still seen 5 to 7 days after the infusion. The changes seen in LDL and triglyceride concentrations in the present study likely represent the result of cessation of statins.

This study was carried out in patients with peripheral vascular disease, a leading cause of morbidity in developed countries and a condition known to be associated with low HDL levels.\(^{5,6}\) This study was not aimed to look at whether rHDL infusion led to a an improvement in claudication symptoms. However, given the results of the ERASE study, this can be partly explained by the higher baseline plaque volume present in the treated group compared to the placebo group, which was not the case in the ERASE study. Plaque characteristics were not analyzed in the study using apoA-I [5].

Thus, we believe that plaque from the superficial femoral artery provides a reasonable indication of what occurs in the coronary circulation. Furthermore, the study was not designed to investigate which component of HDL is responsible for the observed effects: apoA1 or phospholipids.\(^{32,33}\)

\textbf{Conclusion}

In summary, we have shown that a single infusion of HDL results in significant acute changes in plaque morphology. The reduced lipid content and extent of inflammation were consistent with plaque stabilization. Further studies are needed to determine the duration of these effects and whether infusions of reconstituted HDL result in a reduction in clinical events in patients with atherosclerotic disease.

\textbf{Acknowledgments}

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\textbf{Disclosures}

CSL Behring AG (Bern, Switzerland) provided the rHDL but provided no financial support for the study. A.D. owns shares in CSL Ltd.

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