Impact of Short-Term Administration of High-Density Lipoproteins and Atorvastatin on Atherosclerosis in Rabbits

Stephen J. Nicholls, Belinda Cutri, Stephen G. Worthley, Patrick Kee, Kerry-Anne Rye, Shisan Bao, Philip J. Barter

Objective—This study investigates effects of short-term administration of high-density lipoproteins (HDL) and a statin on atherosclerosis in cholesterol-fed rabbits. Effects of HDL apolipoprotein and phospholipid composition have also been investigated.

Methods and Results—Aortic atherosclerosis was established over 17 weeks in 46 rabbits by balloon denudation and cholesterol feeding. During the past 5 days of the cholesterol-feeding period, animals received: (1) no treatment; (2) oral atorvastatin 5 mg/kg on each of the 5 days; or (3) infusions of HDL (8 mg/kg apolipoprotein A-I) on days 1 and 3 of the treatment phase. After euthanization, lesion size and composition were assessed by histological and immunohistochemical analysis. HDL (but not atorvastatin) reduced lesion size by 36% (P<0.05). The ratio of smooth muscle cells to macrophages in the lesions increased 2.6-fold in animals infused with HDL (P<0.05) and 4-fold in those receiving atorvastatin (P<0.01). HDL and atorvastatin reduced matrix metalloproteinase (MMP)-9 expression by 42% (P<0.05) and 45% (P<0.03), respectively. HDL increased thrombomodulin expression 2-fold (P<0.03). The beneficial effects on lesion area and plaque cellular composition were influenced by HDL phospholipid and apolipoprotein composition.

Conclusion—Infusing small amounts of HDL rapidly reduces lesion size and is comparable to atorvastatin in promoting a stable plaque phenotype. (Arterioscler Thromb Vasc Biol. 2005;25:2416-2421.)

Key Words: atherosclerosis ▪ high-density lipoprotein ▪ inflammation ▪ lipoproteins ▪ plaque stabilization

There is overwhelming evidence that lowering the concentration of low density lipoprotein cholesterol (LDL-C) with statins substantially reduces the risk of future cardiovascular events.1,2 Recent studies suggest that statins may also have anti-inflammatory properties.3,4 This supports a potential role of statins in plaque stabilization, and thus in the management of acute coronary syndromes.5 Evidence is mounting that increasing the concentration of high-density lipoproteins (HDL) may be as beneficial as lowering LDL-C levels in terms of reducing the risk of cardiovascular disease.6–8 The best known mechanism underlying an antiatherogenic action of HDL relates to their ability to promote cholesterol efflux from macrophages and foam cells in atherosclerotic lesions and the subsequent delivery of this cholesterol to the liver.9 However, HDL also possess anti-inflammatory10 and anti-oxidant11 properties that may contribute to their cardioprotective properties. This raises the possibility that HDL, like statins, may be protective in acute coronary syndromes.

It has been reported previously that increasing the plasma concentration of HDL reduces the inflammation in established experimental atherosclerosis. Both the transgenic expression of apolipoprotein A-I12 and the infusion of high doses of apolipoprotein A-I Milano as a component of protein:phospholipid complexes13 have been shown to deplete atheroma of macrophages. Given that a macrophage-rich plaque is at increased risk of rupture, which may lead to a subsequent clinical event,14 it is highly likely that a reduction in the inflammatory milieu of the atherosclerotic plaque will be clinically beneficial.

In the current study we have compared the effects of infusing HDL, with those of administering a statin on lesion size and composition in aortic atherosclerosis in rabbits. In addition, reconstituted HDL (rHDL) were infused to assess the influence of HDL apolipoproteins and phospholipids on lesion size and composition.

The results show that infusions of small amounts of HDL rapidly reduce lesion size and have beneficial effects on the atheroma phenotype that are comparable to those of a statin. These findings further strengthen the view that HDL may be important for atherosclerotic plaque stabilization.

Methods

Rabbits

Male New Zealand White rabbits (Institute of Medical and Veterinary Science, Gilles Plains, Australia) aged 12 weeks were main-
tained on a diet comprising 0.2% cholesterol enriched chow (Glen-Forest Stock Feed, Western Australia). All procedures were approved by the Institute of Medical and Veterinary Science Animal Ethics Committee.

**Isolation of HDL**

HDL were isolated from pooled samples of rabbit plasma (Quality Farms of Australia, Lara, VIC, Australia) and from samples of pooled human plasma (Gribbles Pathology, Adelaide, SA, Australia) by sequential ultracentrifugation in the 1.06 to 1.21 g/mL density range. HDL were dialyzed against endotoxin free phosphate-buffered saline (PBS) (pH 7.4, Sigma, St. Louis, Mo) before use.

**Preparation of Reconstituted HDL**

Rabbit apoA-I and human apoA-II were isolated from HDL as previously described. HDL preparations were delipidated, and subjected to anion-exchange chromatography on a Q-Sepharose Fast Flow column attached to an fast protein liquid (FPLC) system (Amersham Biosciences, Uppsala, Sweden). The isolated apolipoproteins were extensively dialyzed against ammonium bicarbonate, lyophilized, and stored at -20°C until used. Lyophilized apolipoproteins were reconstituted in 3 mol/L guanidine hydrochloride and dialyzed against endotoxin free PBS before use.

Discoidal rHDL containing: (1) apoA-I complexed to 1-palmitoyl-2-linoleoyl phosphatidylcholine (PLPC); (2) apoA-I complexed to 1,2-dipalmitoyl phosphatidylcholine (DPPC); or (3) apoA-II complexed to PLPC were prepared using the cholate dialysis method. The final molar ratio of PC/apolipoprotein was 200/1. The phospholipids were chosen to reflect differences in ω-3/ω-6 acyl chain saturation. The resulting rHDL were dialyzed extensively against endotoxin free PBS before use. Protein and phospholipid concentrations were determined by immunoassay and enzymatic assays respectively.

**Establishment of Experimental Atherosclerosis**

Atheroma was induced in 46 rabbits by a combination of a diet containing 0.2% cholesterol-enriched chow and balloon denudation of the abdominal aorta. Balloon denudation was performed 1 week after commencing the cholesterol-enriched diet. All animals consumed the high-cholesterol diet for a further 16 weeks to establish extensive atheroma.

**Experimental Protocol**

At the end of the 16 weeks of cholesterol feeding the animals entered a 5-day treatment phase in which they received intravenous infusions of: (1) rabbit HDL (n=8); (2) rHDL containing apoA-I and PLPC (apoA-I:PLPC, n=6); (3) rHDL containing apoA-I and DPPC (apoA-I:DPPC, n=5); (4) rHDL containing apoA-II and PLPC (apoA-II:PLPC, n=5); or (5) oral atorvastatin (Prizer, Groton, Conn) 5 mg/kg per day administered mixed in the cholesterol enriched chow with 3% peanut oil (n=7); or (6) no treatment (n=15). Infusions of native and rHDL were administered intravenously on days 1 and 3 of the treatment phase. Each infusion contained either 25 mg apoA-I or 31 mg of apoA-II. Atorvastatin was administered throughout the 5-day treatment phase. The high cholesterol diet was continued throughout the treatment phase. On the fifth day of the treatment phase, blood was sampled from a marginal ear vein before animals were euthanized with an overdose of sodium pentobarbital (90 mg/kg, intravenous). The aortic root was cannulated and the aorta was flushed with 500 mL PBS (pH 7.4), followed by perfusion fixation with 500 mL of 4% paraformaldehyde in PBS at 100 mm Hg. After perfusion fixation, the aorta was removed and immersed in fresh fixative.

**Histological Analysis of Lesions**

Specimens were paraffin-embedded, serial 5-μm slices were cut immediately distal to the left renal artery and sections were either subjected to staining with hematoxylin and eosin or used for immunohistochemical analysis. Sections were cut and stained by an investigator who was blinded to the treatment status of the animals. Antibodies applied included mouse monoclonal anti-rabbit RAM11 (DAKO, 1/2000), mouse monoclonal anti-rabbit smooth muscle actin (Sigma, 1/60000), mouse monoclonal anti-rabbit tissue factor (American Diagnostica, 1/500), sheep polyclonal anti-human von Willebrand factor (Binding Site, 1/1500), mouse monoclonal anti-human plasminogen activator inhibitor (PAI)-1 (American Diagnostica, 1/1000), goat polyclonal anti-rabbit thrombomodulin (American Diagnostica, 1/2000), and mouse monoclonal anti-human MMP-9 (Oncogene, 1/200). Digital micrographs of sections were acquired using an Olympus BX40 microscope. Lesion area and the percentage of lesion containing positive staining were determined using ImagePro Plus (Cybernetics). The threshold for positive staining for each antibody was determined and the sections were analyzed by an investigator who was blinded to the treatment status of the animals.

**Plasma Analyses**

Plasma samples were stored at −80°C in EDTA-Na2, until required for analysis. Chemical analyses were performed on a Roche Diagnostics/Hitachi 902 autoanalyzer (Roche Diagnostics GmbH, Mannheim, Germany). Triglyceride concentrations were determined enzymatically. Total cholesterol was determined using a Roche Diagnostics kit. HDL cholesterol was determined by enzymatic assay following precipitation of apolipoprotein B containing lipoproteins with polyethylene glycol. Non-HDL cholesterol was determined as the difference between the values in total plasma and HDL. Rabbit apoA-I concentration was determined by an immunoturbidimetric assay using a sheep anti-rabbit apoA-I immunoglobulin. Human apoA-II was assayed as previously described.

**Data Analysis**

All results are expressed as the mean±standard error of the mean (SEM). Statistical comparisons were made by Student t tests and one way ANOVA, with Bonferroni correction where appropriate, using the statistical program in GraphPad Prism Version 4.0 (GraphPad Software, San Diego, Calif). A value of P<0.05 was considered significant.

**Results**

**Effect of Administering HDL and Atorvastatin on Plasma Lipoproteins**

The concentrations of plasma total cholesterol, triglyceride, HDL cholesterol, and non-HDL cholesterol are presented in the Table. Administration of the cholesterol-enriched diet predictably increased non-HDL-C from 1.2±0.14 mmol/L at baseline to 12.5±2.3 mmol/L at the time of euthanization in the control animals. In the groups of treated animals, whether those receiving infusions of native or reconstituted HDL during the last 5 days of cholesterol feeding or those receiving atorvastatin during this period, the plasma lipid levels (including both HDL-C and non-HDL-C) and apoA-I levels were not statistically different from those in the control animals. There was no detectable apoA-II in any sample, including those from animals infused with the apoA-II rHDL. This presumably reflected the fact that samples were collected at the time of euthanization, 48 hours after the last rHDL infusion.

**Effect of Infusing HDL on Lesion Size**

Compared with cholesterol-fed, untreated animals, infusion of native HDL reduced lesion area by 36% (1.37±0.22 versus 2.13±0.15 mm² in HDL-treated and untreated animals respectively, P<0.05). Administration of atorvastatin did not alter lesion area (2.10±0.36 versus 2.13±0.15 mm² in atorvastatin and untreated animals, respectively) (Figure 1).
Effect of Infusing Native HDL on Lesion Composition

Representative sections of the composition of aortic lesions of animals that received native HDL, atorvastatin, or no treatment are presented in Figure 2. The ratio of smooth muscle cells to macrophages was 2.6-fold higher in the aortas of the HDL-treated animals (12.3±5.9 versus 4.7±0.9 in HDL-treated and untreated animals, respectively; *P*<0.05) and 4-fold higher in atorvastatin treated animals (18.4±7.7 versus 4.7±0.9 in atorvastatin and untreated animals, respectively; *P*<0.01) (Figure 3). This beneficial impact of both HDL and atorvastatin on the SMC to macrophage ratio was derived from the combination of a 39% and 31% increase in SMC with HDL and atorvastatin respectively and a 43% and 54% reduction in macrophages with HDL and atorvastatin respectively (results not shown).

HDL infusion increased lesion expression of thrombomodulin by 112% (10.4±3 versus 4.9±0.7% plaque area in HDL-treated and untreated animals, respectively; *P*<0.03) (Figure 3). Effects of treatment with atorvastatin on thrombomodulin expression were not statistically different from the control. Treatment with atorvastatin or infusion with HDL did not affect PAI-1, tissue factor, and von Willebrand factor expression (results not shown).

MMP-9 expression was decreased by 42% in HDL-treated animals (21.6±7.4 versus 37.3±2.6% plaque area in HDL-treated and untreated animals, respectively; *P*<0.05) and by 45% in atorvastatin-treated animals (20.4±6.2 versus 37.3±2.6% plaque area in atorvastatin-treated and untreated animals, respectively; *P*<0.05) (Figure 3).

Effect of Infusing Reconstituted HDL on Lesion Size

Infusion of rHDL containing apoA-I:PLPC reduced lesion area by 35% (1.39±0.15 versus 2.13±0.15 mm² in rHDL-treated and untreated animals, respectively; *P*<0.05), whereas infusion of apoA-II:PLPC reduced lesion area by 39% (1.29±0.28 versus 2.13±0.15 mm² in rHDL-treated and untreated animals, respectively; *P*<0.05). Lesion area was not significantly reduced by infusing rHDL containing apoA-I:DPPC (1.66±0.28 versus 2.13±0.15 mm² in rHDL-treated and untreated animals, respectively) (Figure 1). This raises the possibility that phospholipid composition may impact on the ability of the rHDL to influence lesion size.

Effect of Infusing Reconstituted HDL on Lesion Composition

Representative sections of the composition of aortic lesions of animals that received rHDL or no treatment are presented in Figure 1 (http://atvb.ahajournals.org). The SMC-to-macrophage ratio was 4.2-fold higher in apoA-I:PLPC rHDL-treated animals (19.7±7.9 versus 4.7±0.9 in apoA-I:PLPC-treated and untreated animals, respectively; *P*<0.01) and 4.1-fold higher in apoA-I:DPPC-treated animals (19.5±10.5 versus 4.7±0.9 in apoA-I:DPPC-treated and untreated animals, respectively; *P*<0.03) (Figure 3). The ratio did not change significantly with infusion of rHDL containing apoA-II:PLPC (7.9±4.9 versus 4.7±0.9 in apoA-II:PLPC-treated and untreated animals, respectively) (Figure 3). Lesion expression of thrombomodulin increased 4.1-fold in apoA-I:PLPC rHDL-treated animals (19.9±6.8 versus 4.9±0.7% plaque area in apoA-I:PLPC-treated and untreated animals, respectively; *P*<0.01), by 2.8-fold in apoA-I:DPPC rHDL-treated animals (13.9±2.1 versus 4.9±0.7% plaque area in apoA-I:DPPC-treated and untreated animals, respectively; *P*<0.01) and by 3.3-fold in apoA-II:PLPC rHDL-treated animals (16.1±4.6 versus 4.9±0.7% plaque area in apoA-II:PLPC-treated and untreated animals, respectively; *P*<0.01) (Figure 3). The lesion content of MMP-9 decreased by 35% in apoA-I:PLPC rHDL-treated animals (24.2±4.8 versus 37.3±2.6% plaque area in apoA-I:PLPC-treated and untreated animals, respectively; *P*<0.05) and by 60% in apoA-II:PLPC rHDL-treated animals (15.1±4.2 versus 37.3±2.6% plaque area in apoA-II:PLPC-treated and untreated animals, respectively; *P*<0.05) (Figure 3).

### Table: Plasma Lipid Profiles of Animals at the Time of Euthanization

<table>
<thead>
<tr>
<th></th>
<th>TC</th>
<th>TG</th>
<th>HDL-C</th>
<th>Non-HDL-C</th>
<th>ApoA-I</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>12.9±2.2</td>
<td>0.74±0.22</td>
<td>0.40±0.09</td>
<td>12.5±2.3</td>
<td>580±170</td>
</tr>
<tr>
<td>HDL</td>
<td>12.4±4.22</td>
<td>1.1±0.56</td>
<td>0.38±0.04</td>
<td>12.0±4.2</td>
<td>nd</td>
</tr>
<tr>
<td>Atorvastatin</td>
<td>11.1±3.8</td>
<td>0.88±0.40</td>
<td>0.30±0.05</td>
<td>10.8±4.1</td>
<td>770±150</td>
</tr>
<tr>
<td>apoA-I:PLPC rHDL</td>
<td>9.7±2.8</td>
<td>0.47±0.19</td>
<td>0.34±0.04</td>
<td>9.4±4.0</td>
<td>610±190</td>
</tr>
<tr>
<td>apoA-I:DPPC rHDL</td>
<td>15.3±3.0</td>
<td>0.96±0.55</td>
<td>0.30±0.06</td>
<td>15.0±4.2</td>
<td>540±300</td>
</tr>
<tr>
<td>apoA-II:PLPC rHDL</td>
<td>12.9±3.4</td>
<td>0.40±0.14</td>
<td>0.29±0.08</td>
<td>12.6±5.0</td>
<td>nd</td>
</tr>
</tbody>
</table>

Results expressed as mmol/L (mean±SEM) for the lipids and mg/L for the apoA-I. nd indicates not determined; TC, total cholesterol; TG, triglyceride.

There were no significant differences between treatment groups.
treated animals, respectively; \(P<0.01\). MMP-9 content was not altered by infusing apoA-I:DPPC rHDL (37.8±5.5 versus 37.3±2.6% plaque area in apoA-I:DPPC-treated and untreated animals, respectively; NS) (Figure 3).

**Discussion**

The study reported here provides the first direct comparison of the effects of HDL infusion and statin administration on the size, morphology, and composition of atherosclerotic plaques. It was found that infusions of HDL were as effective as a statin in promoting a more stable plaque phenotype and more effective than a statin in reversing the size of the lesion in this experimental model of atherosclerosis. Infusion of either native HDL or rHDL given on 2 occasions during the last 5 days of a 17-week induction of atherosclerosis, promoted not only a significant reduction in lesion size but also an increased ratio of SMC to macrophages. HDL infusion also promoted favorable changes in expression of MMP-9 and thrombomodulin, a finding that extends earlier observations made in vitro that HDL reduce MMP release from monocytes\(^{23}\) and are antithrombotic\(^{24}\).

The observed reduction in atherosclerosis lesion size after HDL infusion is consistent with previous studies using both rHDL\(^{25}\) and native HDL\(^{13,26}\) in animals and humans.\(^6\) The current study extends these findings not only by the comparison with atorvastatin but also by asking whether these effects are modulated by the phospholipid or apolipoprotein composition of the HDL.

These effects were achieved with infusions of relatively small amounts of the HDL preparations. Rabbits received 2 infusions of HDL each containing 25 mg apoA-I (or apoA-II). A 3-kg rabbit with a plasma volume of \(\approx120\text{ mL}\) and an apoA-I concentration of \(\approx600\text{ mg/L}\) (Table) would have a plasma apoA-I pool size of \(\approx70\text{ mg}\). This would have been increased by 25 mg (\(\approx30\%\)) immediately after the infusion. Given a fractional catabolic rate of apoA-I in rabbits of 0.8 pools/d,\(^{27}\) the apoA-I concentration at the time of euthanization, 48 hours after the final infusion, would almost certainly have returned to pretreatment levels. In the case of apoA-II, it should be remembered that rabbits are naturally deficient in apoA-II. Infusing 3kg rabbits with of rHDL containing 31 mg of human apoA-II would have resulted in a concentration of apoA-II of \(\approx250\text{ mg/L}\) (comparable to the concentration of apoA-II in human plasma) immediately after completion of the infusion. The rate at which the apoA-II is removed from rabbit plasma is not known, although it was virtually all gone from the plasma at the time of euthanization of the rabbits 48 hours after the second of the 2 infusions.
The observation that the effects of HDL on lesion size and composition were achieved by a transient 30% increase in apoA-I concentration suggests that infusing HDL has effects that may extend beyond those resulting from a simple increase in the concentration of plasma HDL. These effects may relate to both promotion of cholesterol efflux from the arterial wall and a number of other actions including inhibition of inflammation and oxidative stress.

Overall, the beneficial effects of the rHDL were comparable to those of native HDL in both reducing lesion size and promoting a more stable plaque phenotype. The effectiveness of the rHDL was, however, influenced by the particle composition. Changing the phospholipid from PLPC to DPPC may have compromised the ability of the rHDL to decrease lesion size, with the reduction in lesion size following infusion of DPPC rHDL not reaching statistical significance, although this may have reflected no more than the relatively small numbers of animals in the group. Substituting apoA-II for apoA-I in rHDL had no effect on the ability of the rHDL to reduce lesion size although it may have reduced the plaque stabilizing properties of the particles.

The effect of phospholipid composition on the ability of rHDL to promote cholesterol efflux is well-documented, with good evidence that particles containing phospholipids with unsaturated fatty acid chains are superior as acceptors of cell cholesterol to those that contain increasing amounts of saturated fatty acid chains. Changing the phospholipid composition of HDL alters its ability to promote cholesterol efflux from cultured fibroblasts in vitro, as well as in vivo. This effect has been proposed to result from an inverse relationship between the fluidity and saturation of the HDL phospholipid acyl chains.

Effects of PLPC and DPPC containing rHDL on arterial cell morphology have been reported in a rabbit model in which acute vascular inflammation was induced by insertion of a nonocclusive peri-arterial collar. In those studies the collar-induced, acute inflammation (as assessed by neutrophil infiltration) was inhibited to a similar extent by rHDL containing PLPC and DPPC. Thus, it is possible that the phospholipid composition of HDL impacts on some but not all properties of the particles. Given that HDL phospholipid composition varies in response to changes in the composition of dietary fat, the phenomenon is of potential clinical importance and worthy of further investigation.

Changing the apolipoprotein composition of the rHDL impacted on some, but not all, of the effects of the rHDL infusions. Replacement of apoA-I with apoA-II did not impact on the ability of rHDL to reduce lesion size or to promote favorable changes in thrombomodulin and MMP expression. The ability of the rHDL to increase the SMC to macrophage ratio, however, was diminished. It is possible that the reduction in lesion size promoted by the apoA-II-containing rHDL is secondary to the ability of the particles to promote cholesterol efflux. An impaired ability of apoA-II-containing rHDL to improve the cellular morphology of lesions may reflect a reduced anti-inflammatory function of the apoA-II-containing particles. Some human population studies and transgenic animal studies have raised the possibility that HDL containing apoA-I without apoA-II may be superior to HDL that contain both apoA-I and apoA-II in their ability to protect against atherosclerosis. Other studies, however, have suggested that the protection conferred by apoA-I-containing HDL and apoA-II-containing HDL is comparable.

One limitation of this study relates to the relatively small numbers of animals in each group. However, despite this, the effects of infusing HDL on lesion size and composition were profound and were, in almost all cases, statistically significant. Having established in this rabbit model that short term infusions of HDL, whether native or reconstituted, are as effective as administration of a statin in promoting a stable plaque phenotype and are more effective than a statin in reducing the size of the lesion, 2 obvious questions arise: will the combination of the 2 therapies be additive (or even synergistic) in the management of atherosclerosis, and what is the relevance of the results to the management of humans with atherosclerosis? The issue of the effects of the combination will be tested in future studies in the rabbit model of atherosclerosis. The relevance of the findings to the management of human atherosclerosis, however, will require clinical designed trials to investigate the effect of HDL-raising (whether by infusing rHDL or by administration of a CETP.
inhibitor) on atheroma burden and clinical events when given on a background of statin therapy.

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