Ability of reconstituted high density lipoproteins to inhibit cytokine-induced expression of vascular cell adhesion molecule-1 in human umbilical vein endothelial cells

Paul W. Baker,[1,*,†] Kerry-Anne Rye,[†,§] Jennifer R. Gamble,[†] Mathew A. Vadas,[†] and Philip J. Barter[*,†,§]

University Department of Medicine,* Royal Adelaide Hospital, Adelaide, Australia; Hanson Centre,[†] Institute of Medical and Veterinary Science, Adelaide, Australia; Cardiovascular Investigation Unit,[§] Royal Adelaide Hospital, Adelaide, Australia

Abstract **Previous studies have shown that both high density lipoproteins (HDL) isolated from human plasma and reconstituted HDL (rHDL) are effective inhibitors of adhesion molecule expression in human endothelial cells. In this study rHDL have been used to investigate whether HDL particle shape, size, apolipoprotein composition or lipid composition are important determinants of the ability of HDL to inhibit the TNF-α induced expression of vascular cell adhesion molecule-1 (VCAM-1) in human umbilical vein endothelial cells (HUVECs). On the basis of these studies it is possible to draw several firm conclusions.** ***i*****) Neither phospholipid-containing vesicles nor lipid-free apolipoprotein (apo) A-I inhibit VCAM-1 expression in HUVECs.** ***ii*****) Simple discoidal complexes containing only phospholipid and apoA-I (discoidal (A-I)rHDL) are sufficient to inhibit the TNF-α-induced expression of VCAM-1 in HUVECs.** ***iii*****) Spherical apoA-I-containing rHDL (spherical (A-I)rHDL) are superior to discoidal (A-I)rHDL as inhibitors.** ***iv*****) The particle size of spherical (A-I)rHDL has no influence on the inhibition.** ***v*****) Spherical rHDL that contain apoA-I are superior as inhibitors of VCAM-1 to those containing apoA-II when the rHDL preparations are equated for apolipoprotein molarity. However, when compared at equivalent particle molarities, this difference is no longer apparent.** ***vi*****) Replacement of cholesteryl esters with triglyceride in the core of spherical (A-I)rHDL has no effect on the ability of these particles to inhibit VCAM-1 expression. From these results it is tempting to speculate that variations in inhibitory activity may contribute to the variations observed in the anti-atherogenicity of different HDL subpopulations.—** Baker, P. W., K-A. Rye, J. R. Gamble, M. A. Vadas, and P. J. Barter. **Ability of reconstituted high density lipoproteins to inhibit cytokine-induced expression of vascular cell adhesion molecule-1 in human umbilical vein endothelial cells.** ***J. Lipid Res.*** **1999.** 40: **345–353.**

Supplementary key words reconstituted HDL • endothelial cells • VCAM-1 • phospholipids • apolipoproteins

High density lipoproteins (HDL) are well known to protect against the development of coronary heart disease (1). This fact has stimulated considerable investigation into the functions of HDL and the potential mechanisms by which these lipoproteins inhibit atherosclerosis. Several mechanisms have been proposed. These include the involvement of HDL in reverse cholesterol transport (2–4) the ability of HDL to protect against lipid peroxidation (5) and more recently, the ability of HDL to inhibit the expression of adhesion molecules in endothelial cells (6, 7).

Human HDL have been shown to inhibit the cytokine-induced expression of vascular cell adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-1, and E-selectin in human umbilical vein endothelial cells (HUVECs) (6). Because endothelial cell expression of each of these adhesion molecules has been implicated in the recruitment of monocytes into the arterial intima in what appears to be a key early step in the initiation of atherosclerosis, the ability of HDL to inhibit their expression has clear implications in terms of a possible mechanism by which HDL may protect against the development of coronary heart disease.

This study focuses specifically on the ability of HDL to inhibit the TNF-α-induced expression of endothelial cell VCAM-1. The expression of VCAM-1 coincides with early development of foam cell lesions in hypercholesterolemic rabbits (8). It has also been detected in the arterial endothelium over existing human atheromatous plaques (9).

Abbreviations: HDL, high density lipoproteins; rHDL, reconstituted high density lipoproteins; LDL, low density lipoproteins; apo, apolipoprotein; (A-I)rHDL, rHDL containing apoA-I; (A-II)rHDL, rHDL containing apoA-II; LCAT, lecithin:cholesterol acyltransferase; CETP, cholesteryl ester transfer protein; TBS, Tris-buffered saline; VCAM-1, vascular cell adhesion molecule-1; HUVEC, human umbilical vein endothelial cell; TNF, tumour necrosis factor; PL, phospholipid; UC, unesterified cholesterol; CE, cholesteryl ester; TG, triglyceride; POPC, β-oleoyl-γ-palmitoyl-l-α-phosphatidylcholine; SUV, small unilamellar vesicles; PC, phosphatidylcholine; FPLC, fast protein liquid chromatography.

[1] To whom correspondence should be addressed.

Furthermore, the level of circulating VCAM-1 in serum correlates positively with the extent of angiographically determined atherosclerosis in humans (10).

The HDL in human plasma include several distinct subpopulations of particles that vary in size, shape (discoidal and spherical), and in the composition of both their lipid and apolipoprotein components. It is not known whether the ability of HDL to inhibit endothelial cell adhesion molecule expression is influenced by differences in the physical and chemical characteristics of these particles. Other studies from this laboratory have shown that there is considerable variation in the ability of HDL from different subjects to inhibit VCAM-1 expression (7). To this end, we have investigated whether particle size, shape, apolipoprotein composition, and core lipid composition influence the ability of HDL to inhibit the TNF-α-induced expression of VCAM-1 in human umbilical vein endothelial cells (HUVECs).

HDL isolated from human plasma (native HDL) could not be used for these studies because of their intrinsic heterogeneity. There are currently no techniques capable of resolving native HDL into distinct subpopulations of particles that are homogeneous in terms of size, shape, or composition. For this reason we have chosen to use reconstituted HDL (rHDL) in which the size, shape, apolipoprotein composition, and core lipid composition can be tailored to demand and strictly controlled. Hence, they are ideal tools for investigating the effects of changes in these parameters on adhesion molecule expression. Moreover, we and others have already demonstrated that rHDL, like their native counterparts, are able to inhibit the cytokine-induced expression of adhesion molecules by endothelial cells (6, 11, 12).

MATERIALS AND METHODS

Isolation of apoA-I and apoA-II

HDL were isolated by ultracentrifugation, delipidated by a standard procedure (13), and subjected to anion-exchange chromatography on an XK 26/40 column containing Q Sepharose Fast Flow gel attached to a fast protein liquid chromatography (FPLC) system (Pharmacia LKB Biotechnology, Uppsala, Sweden) (14). After chromatography the apolipoproteins were dialyzed extensively (3 × 5 liter) against 20 mm NH_4HCO_3, lyophilized, and stored at −20°C. When required, the apolipoproteins were reconstituted by dissolving in 10 mm Tris-HCl buffer (pH 8.2) containing 3 m guanidine-HCl and 0.01% (w/v) EDTA-Na_2, after which they were extensively dialyzed (5 × 1 liter) against 10 mm Tris-buffered saline (TBS, pH 7.4) containing 150 mm NaCl, 0.005% (w/v) EDTA-Na_2, and 0.006% (w/v) NaN_3.

Isolation of CETP and LCAT

Cholesteryl ester transfer protein (CETP) was isolated from pooled human plasma by ammonium sulfate fractionation, ultracentrifugation, and sequential chromatography as described previously (15). Lecithin:cholesterol acyltransferase (LCAT) was also isolated from pooled human plasma as described previously (16).

Preparation of small unilamellar vesicles

Small unilamellar vesicles (SUVs) containing β-oleoyl-γ-palmitoyl-l-α-phosphatidylcholine (POPC) were prepared as described by Jonas (17). Briefly, POPC (12 mg) was placed in a large glass test tube to which chloroform–methanol (1:1 v/v, 500 μl) was added. The POPC was then dispersed as a thin film onto the walls of the tube before drying under nitrogen for 2 h at 40°C. After drying, endotoxin-free PBS (1 ml) was added and the solution was vortexed to obtain multilamellar vesicles (MLVs). The MLVs were then sonicated at 4°C (Sonifier B12, Setting 1 to 2, Branson Sonic Power Company, Danbury, CT) with a microtip sonic probe in 3-min bursts followed by 1- to 2-min intervals (total of 30 min sonication, i.e., 10 × 3 min bursts). The resultant clear solution of SUVs was then centrifuged in a microfuge (10 min, 15,000 rpm) to remove titanium fragments.

Preparation of discoidal (A-I)rHDL

Discoidal (A-I)rHDL containing POPC and apoA-I (molar ratio 100:1) were prepared by the cholate dialysis method (18). These particles were compared with lipid-free apoA-I, phospholipid (PL) SUVs, and spherical (A-I)rHDL in terms of their ability to inhibit cytokine-induced VCAM-1 expression in HUVECs.

Preparation of spherical (A-I)rHDL

Discoidal (A-I)rHDL containing POPC, unesterified cholesterol (UC), and apoA-I (molar ratio 100:10:1) were prepared by the cholate dialysis method (18). The resulting discoidal (A-I)rHDL were converted to spheres by incubation for 24 h at 37°C with LDL, LCAT, bovine serum albumin (fatty acid-free), and β-mercaptoethanol as described previously (19). The spherical (A-I)rHDL were then isolated by sequential ultracentrifugation in the density range $1.07 < d < 1.21$ g/ml with two spins at the lower density (24 h, Ti 55.2 rotor, 55,000 rpm, 4°C) and a single spin at the higher density (18 h, Ti 100.4 rotor, 100,000 rpm, 4°C).

Preparation of spherical (A-II)rHDL

Spherical (A-II)rHDL were prepared by displacing apoA-I from spherical (A-I)rHDL with lipid-free apoA-II during a 1-h incubation at room temperature. Conditions used were such that each molecule of apoA-I was displaced by two molecules of lipid-free apoA-II as described previously (19). Control incubations contained spherical (A-I)rHDL with TBS in place of lipid-free apoA-II. The spherical (A-II)rHDL were then isolated by ultracentrifugation at 1.21 g/ml (18 h, Ti 100.4 rotor, 100,000 rpm, 4°C).

Preparation of small spherical (A-I)rHDL containing either cholesteryl ester or triglyceride in their core

Small spherical (A-I)rHDL containing cholesteryl esters in their core were prepared by incubating spherical (A-I)rHDL for 24 h at 37°C with CETP as described previously (15). If required, control preparations of spherical (A-I)rHDL were incubated in parallel with TBS in the absence of CETP. The small spherical rHDL containing cholesteryl esters in their core were then isolated by sequential ultracentrifugation in the density range $1.07 < d < 1.25$ g/ml with one spin at each density (18 h, Ti 100.4 rotor, 100,000 rpm, 4°C). As reported previously, the unmodified spherical (A-I)rHDL contained three molecules of apoA-I per particle, while the small spherical (A-I)rHDL contained two molecules of apoA-I per particle (15).

Small triglyceride core-enriched spherical (A-I)rHDL were prepared by incubating spherical (A-I)rHDL for 24 h at 37°C with CETP and Intralipid (Kabi Pharmacia AB, Sweden) as described previously (20). These small triglyceride core-enriched rHDL were then isolated by sequential ultracentrifugation in the density range $1.07 < d < 1.25$ g/ml with two spins at the lower density (18 h, Ti 100.4 rotor, 100,000 rpm, 4°C) and a single spin at the higher density (18 h, Ti 100.4 rotor, 100,000 rpm, 4°C). The small triglyceride core-enriched rHDL each contained two molecules of apoA-I per particle (20).

General notes on rHDL preparation

After sequential ultracentrifugation all rHDL were dialyzed against TBS (3 × 1 liter). If required, rHDL were concentrated by centrifugation (~10 min, 1500 rpm, 4°C) using CF25 Centriflo ultrafiltration membrane cones (Amicon, Victoria, Australia). Before addition to HUVECs, all rHDL were dialyzed over 24 h against endotoxin-free PBS (3 × 1 liter).

HUVEC isolation, culture and incubation conditions

HUVECs were isolated as described previously (21). Cells were cultured on gelatin-coated culture flasks in medium M199 with Earles Salts (Trace Biosciences, Australia) supplemented with 20% fetal calf serum (Commonwealth Serum Laboratories, Melbourne, Australia), 20 mm HEPES, 2 mm glutamine, 1 mm sodium pyruvate, nonessential amino acids, 12 μg/ml penicillin, 16 μg/ml gentamicin, 20 mg/ml endothelial growth supplement (Collaborative Research, Australia), and 20 mg/ml heparin (Sigma) (referred to as complete media). Confluent preparations of passage 2, 3, or 4 HUVECs were washed with 10 mm EDTA-Na_2 (5 ml) in phosphate-buffered saline (PBS), trypsinized, and re-plated in complete media (as described above) onto 24-well gelatin-coated plates at a density of 3×10^6 cells/ml (500 μl/well). After a 5-h re-attachment period, a portion of the complete media (100 μl) was removed and replaced with PBS alone (100 μl) or with rHDL in PBS (100 μl). The cells were then pre-incubated for 16 h after which time tumor necrosis factor (TNF)-α (100 U/ml) was added to the culture medium. After a further 5 h the cell surface expression of VCAM-1 was measured by flow cytometry. Native HDL_3 at concentrations of 1, 2, and 4 μm apoA-I was used as a positive control in every experiment.

Flow cytometry analysis of VCAM-1

Cell surface expression of VCAM-1 was determined as described previously (6). In brief, the cells were washed with FACS wash (RPMI 1640, containing 10 mm HEPES, 3.1 mm NaN_3, and 2.5% fetal calf serum), incubated with mouse monoclonal antibody to VCAM-1 (51-10C9) for 30 min at 4°C, washed again with FACS wash, and incubated for a further 30 min at 4°C with FITC-conjugated secondary antibody (Immunotech FITC conjugated $F(ab)_2$ fragment goat (mouse IgG)). The cells were then washed twice with PBS and harvested by incubation for 30 sec at room temperature with trypsin; at 30 sec FACS wash was added to neutralize the trypsin. The cells were pelleted by centrifugation in an Immunfuge and the pellet was resuspended in FACS fixative (PBS containing 111 mm glucose, 3.1 mm NaN_3, and 350 mm formaldehyde). The expression of VCAM-1 was then measured as fluorescence intensity using an Epics XL-MCL flow cytometer (Coulter, Hialeah, FL). Each sample counted 10,000 cells. Controls included replacement of VCAM-1 primary antibody with an isotype-matched non-relevant antibody, and exclusion of TNF-α.

Statistical analysis

All statistical tests (Two-Sample *t*-test: Assuming Equal Variance and ANOVA: Two-Factor with Repeated Measures) were performed using the data analysis package in Microsoft Excel, Version 7.0. Analysis of variance was performed by treating all points within a data set as a group; points were not treated individually. Furthermore, points that were not paired (i.e., did not have a corresponding point in the opposing data set) were excluded from the analysis. Significance was determined at the 95% confidence interval, i.e., $P < 0.05$. All results are expressed as mean ± SEM (n = 4, unless specified).

RESULTS

Comparison of the inhibitory activities of lipid-free apoA-I, phospholipid vesicles, and discoidal (A-I)rHDL (Fig. 1)

HUVECs were pre-incubated for 16 h with either *i*) lipid-free apoA-I, *ii*) POPC-containing SUVs, or *iii*) discoidal rHDL complexes containing apoA-I and POPC in a molar ratio of 1:100. The cells were then activated by TNF-α and the expression of VCAM-1 was measured 5 h later. Neither lipid-free apoA-I at concentrations up to 32 μm (■) nor the POPC-containing SUVs at PL concentrations up to 3200 μm (○) had a significant effect on VCAM-1 expression (Fig. 1). In contrast, when apoA-I and POPC were complexed into discoidal rHDL (●), there was a significant concentration-dependent inhibition of the cytokine-induced expression of VCAM-1 (Fig. 1). When discoidal rHDL were present at an apoA-I concentration of 32 μm (PL concentration of 3200 μm) VCAM-1 expression was inhibited by about 30%. It appears therefore, that neither PL nor lipid-free apoA-I alone is sufficient to achieve inhibition of adhesion molecule expression with inhibition only being observed when both components are presented to HUVECs in the form of a lipoprotein complex.

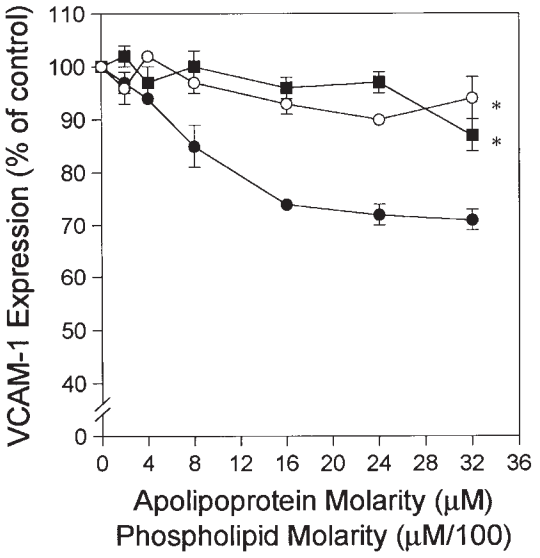

Fig. 1. The effect of lipid-free apoA-I, POPC vesicles, and discoidal (A-I)rHDL on the TNF-α induced expression of VCAM-1 in HUVECs. Small unilamellar vesicles (SUVs) of POPC and discoidal (A-I)rHDL containing POPC and apoA-I in a molar ratio of 100:1 were prepared as described in Methods. HUVECs were pre-incubated with lipid-free apoA-I, POPC SUVs, or discoidal (A-I)rHDL for 16 h before stimulation of the cells with TNF-α. VCAM-1 expression was determined 5 h after stimulation with TNF-α. The apolipoprotein molarity shown on the horizontal axis refers to both the lipid-free apoA-I and the rHDL, while the phospholipid molarity refers to both the SUVs and the rHDL. Results are expressed as means ± SEM (n = 6); lipid-free apoA-I (■), POPC SUVs (○), and discoidal (A-I)rHDL (●). *Significant difference from discoidal (A-I)rHDL by ANOVA, $P < 0.05$. All paired points (i.e., points that had a corresponding point in the opposing data set) were treated as a group and used for the analysis of variance; points that were not paired were excluded from the analysis.

Effect of particle shape on the inhibitory activity of rHDL (Fig. 2)

HUVECs were pre-incubated for 16 h with either discoidal (A-I)rHDL (containing two molecules of apoA-I per particle) or spherical (A-I)rHDL (containing three molecules of apoA-I per particle) (**Table 1**) before activating the cells with TNF-α. The incubation was then continued for another 5 h before the cells were assayed for VCAM-1 expression. When the discoidal and spherical (A-I)rHDL were equated in terms of apoA-I molarity, it was apparent that the spherical particles (●) were more effective inhibitors of VCAM-1 expression than the discoidal particles (○) ($P < 0.05$) (Fig. 2a). Figure 2b shows the same experiment but with the discs and spheres now equated in terms of particle molarities which were calculated on the basis of the discoidal and spherical (A-I)rHDL containing, respectively, two and three molecules of apoA-I per particle. When equated for rHDL particle molarity, the superiority of the spheres over the discs as inhibitors of VCAM-1 expression was even more pronounced ($P < 0.05$). At a particle molarity of 5.33 μm, spherical (A-I)rHDL inhibited VCAM-1 expression by 34% while the corresponding concentration of discoidal (A-I)rHDL achieved only a 10% inhibition (Fig. 2b).

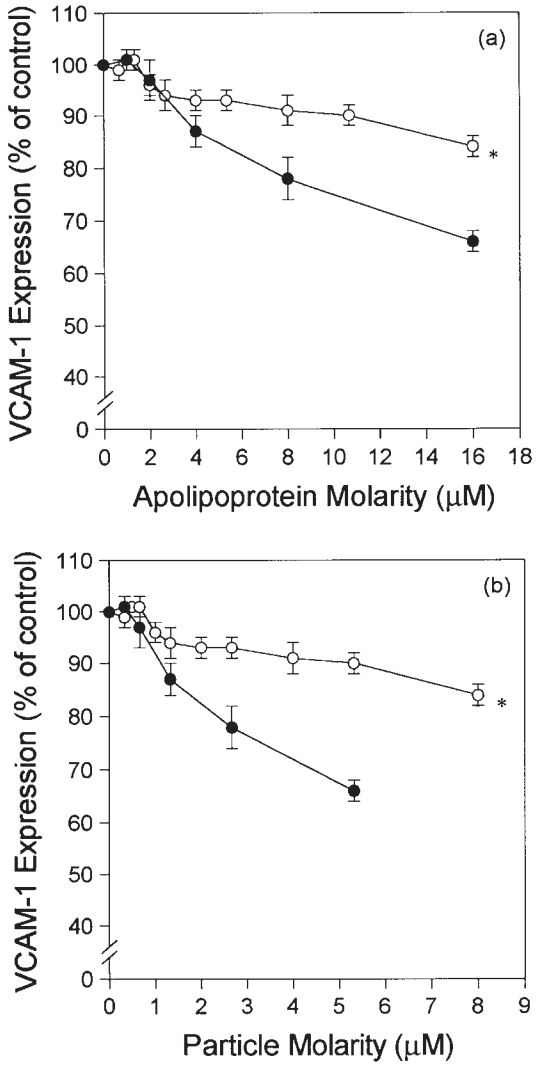

Fig. 2. The effect of (A-I)rHDL particle shape on the TNF-α induced expression of VCAM-1 in HUVECs. Spherical and discoidal (A-I)rHDL were prepared as outlined in Table 1. HUVECs were pre-incubated with spherical or discoidal (A-I)rHDL for 16 h before stimulation of the cells with TNF-α. VCAM-1 expression was determined 5 h after stimulation with TNF-α. Apolipoprotein molarity was converted to rHDL particle molarity on the basis of the fact that the spherical (A-I)rHDL contained three apoA-I molecules per particle while the discoidal (A-I)rHDL contained two apoA-I molecules per particle (Table 1). Results are expressed as means ± SEM (n = 5); spherical (A-I)rHDL (●) and discoidal (A-I)rHDL (○). *Significant difference from spherical (A-I)rHDL by ANOVA, $P < 0.05$. All paired points (i.e., points that had a corresponding point in the opposing data set) were treated as a group and used for the analysis of variance; points that were not paired were excluded from the analysis.

TABLE 1. Size and composition of discoidal and spherical (A-I)rHDL

rHDL Species	Stokes Diameter[a]	Composition[b]			
		A-I	PL	CE	UC
	nm	*mol/mol rHDL*			
Discoidal (A-I)	9.9	2.0	196.3	—	—
Spherical (A-I)	9.5	3.0	100.7	54.3	12.7

Discoidal (A-I)rHDL were prepared from apoA-I and β-oleoyl-γ-palmitoyl-l-α-phosphatidylcholine (POPC) by the cholate dialysis method as outlined in Methods. Spherical (A-I)rHDL were prepared by incubating discoidal (A-I)rHDL with LCAT and LDL also as outlined in Methods. The number of moles of apoA-I per mole of rHDL was determined by cross-linking (15). The number of moles of the other constituents, relative to that of apoA-I, was determined by compositional analysis; A-I, apoA-I; PL, phospholipid; CE, cholesteryl ester; UC, unesterified cholesterol.

[a] Determined by nondenaturing gradient gel electrophoresis.

[b] Values represent the means of triplicate determinations on three independent rHDL preparations.

Effect of particle size on the inhibitory activity of spherical (A-I)rHDL (Fig. 3)

Preparations of spherical (A-I)rHDL (diameter 9.4 nm with three molecules of apoA-I per particle) were converted into small particles (diameter 7.8 nm with two molecules of apoA-I per particle) (**Table 2**) by incubation with CETP as outlined in Methods. The unmodified spherical (A-I)rHDL and the modified, small spherical (A-I)rHDL were then compared as inhibitors of the cytokine-induced expression of VCAM-1 in HUVECs. There was no significant difference between the unmodified (●) and small spherical (A-I)rHDL (○) in terms of their inhibitory activities whether the comparison was made on the basis of apolipoprotein molarity (Fig. 3a) or rHDL particle molarity (Fig. 3b).

Effect of apolipoprotein composition on the inhibitory activity of spherical rHDL (Fig. 4)

Aliquots of spherical (A-I)rHDL (three molecules of apoA-I per particle) were converted into spherical (A-II)rHDL (six molecules of apoA-II per particle) as outlined in Methods. The size and composition of these rHDL are presented in **Table 3**. The apolipoprotein-specific preparations of spherical rHDL were then compared in terms of their ability to inhibit the TNF-α-induced expression of

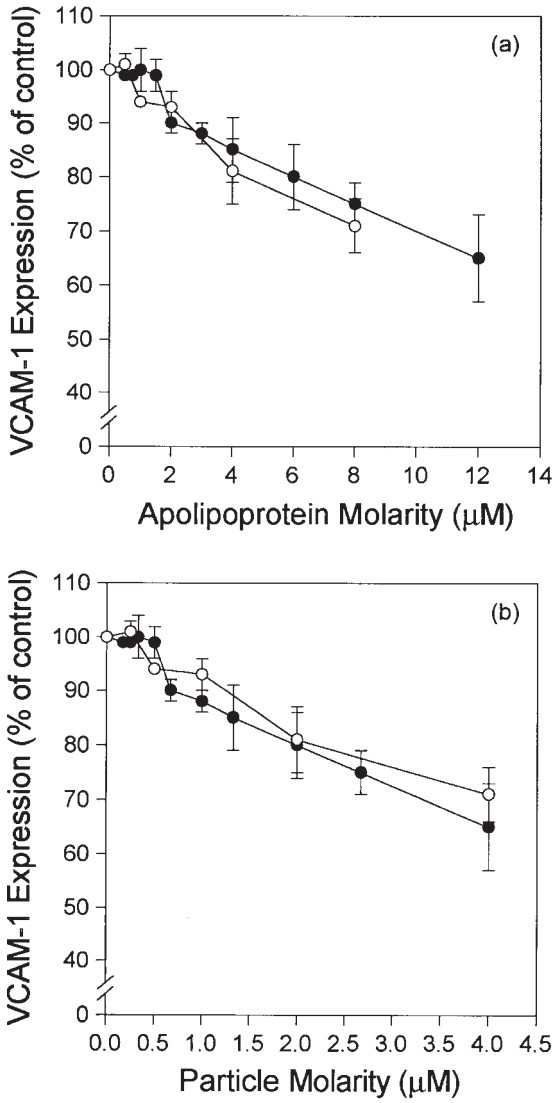

Fig. 3. The effect of the size of spherical (A-I)rHDL on the TNF-α induced expression of VCAM-1 by HUVECs. Unmodified and small spherical (A-I)rHDL were prepared as outlined in Table 2. HUVECs were pre-incubated with unmodified or small spherical (A-I)rHDL for 16 h before stimulation of the cells with TNF-α. VCAM-1 expression was determined 5 h after stimulation with TNF-α. Apolipoprotein molarity was converted to rHDL particle molarity on the basis of the fact that the unmodified spherical (A-I)rHDL contained three apoA-I molecules per particle while the small spherical (A-I)rHDL contained two apoA-I molecules per particle (Table 2). Results are expressed as mean ± SEM (n = 4); unmodified spherical (A-I)rHDL (●) and small spherical (A-I)rHDL (○).

VCAM-1 in HUVECs. When compared on the basis of apolipoprotein molarity (Fig. 4a), spherical (A-I) rHDL (●) were clearly superior to spherical (A-II)rHDL (○) as inhibitors of VCAM-1 expression. However, when equated in terms of particle molarity, the difference between spherical (A-I)rHDL or spherical (A-II)rHDL was no longer apparent (Fig. 4b).

Effect of core lipid composition on the inhibitory activity of spherical (A-I)rHDL (Fig. 5)

In order to investigate the effect of core lipid composition on the ability of spherical rHDL to inhibit VCAM-1 expression by HUVECs, preparations of spherical (A-I)rHDL (initially with a core consisting solely of cholesteryl esters and a surface containing three molecules of apoA-I per particle) were modified during incubations with either CETP alone (●) or with CETP plus Intralipid (○) as described in Methods. These incubation conditions, respectively, generated small spherical (A-I)rHDL (with two apoA-I molecules per particle) in which the core lipid was either cholesteryl ester or considerably enriched in triglyceride (**Table 4**). The enrichment of spherical (A-I)rHDL core lipid with triglyceride had no effect on the ability of these particles to inhibit TNF-α-induced VCAM-1 expression in HUVECs (Fig. 5).

TABLE 2. Size and composition of unmodified and small spherical (A-I)rHDL

rHDL Species	Stokes Diameter	Composition[a]			
		A-I	PL	CE	UC
	nm	*mol/mol rHDL*			
Spherical (A-I)	9.4	3.0	99.9	60.9	10.1
Small spherical (A-I)	7.8	2.0	59.7	33.8	4.5

The spherical (A-I)rHDL were prepared as outlined in the legend to Table 1. The small spherical (A-I)rHDL were prepared by incubating spherical (A-I)rHDL with CETP as described in Methods. The number of moles of apoA-I per mole of rHDL was determined by cross-linking (15). The number of moles of the other constituents, relative to that of apoA-I, was determined by compositional analysis; A-I, apoA-I; PL, phospholipid; CE, cholesteryl ester; UC, unesterified cholesterol.

[a] Values represent means of triplicate determinations on two independent rHDL preparations.

DISCUSSION

The ability of HDL to inhibit the cytokine-induced expression of adhesion molecules in endothelial cells has been well documented (6, 11, 12) although the mechanism of the inhibition is still not known. The present studies were designed, therefore, to investigate how the morphology and composition of HDL influence their inhibitory activity and to gain some insight into possible mechanisms involved.

The original studies showing that HDL inhibit the expression of endothelial cell adhesion molecules were conducted with native HDL (6, 7) which are known to include several subpopulations of particles of differing size, shape, and composition. In this study homogeneous preparations of rHDL of defined particle shape, size, and composition have been used to investigate how these physical and chemical characteristics impact on the inhibitory activity of the particles. As in the earlier studies with native HDL (6, 7), the concentrations of rHDL used in the present study were within the physiological range of HDL in plasma. On the basis of these studies it is possible to draw several firm conclusions. *i*) Simple discoidal complexes containing phospholipid and apoA-I only, but not lipid-free apoA-I alone or phospholipid alone, are sufficient to inhibit the TNF-α-induced expression of VCAM-1 by HUVECs. *ii*) Spherical (A-I)rHDL are superior to dis-

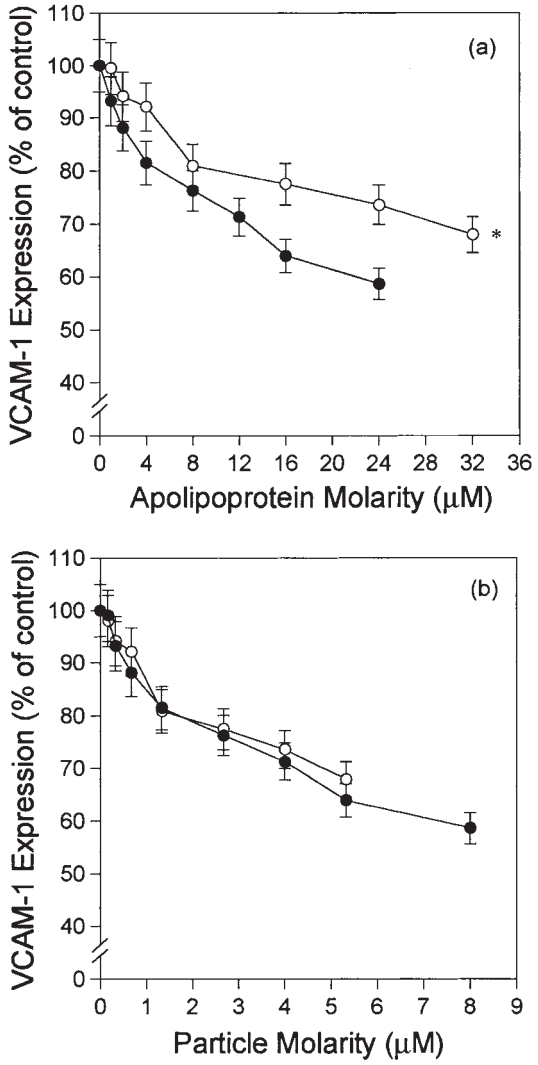

Fig. 4. The effect of apolipoprotein composition of spherical rHDL on the TNF-α induced expression of VCAM-1 in HUVECs. Spherical (A-I)rHDL and (A-II)rHDL were prepared as outlined in Table 3. HUVECs were pre-incubated with spherical (A-I)rHDL or (A-II)rHDL for 16 h before stimulation of the cells with TNF-α. VCAM-1 expression was determined 5 h after stimulation with TNF-α. Apolipoprotein molarity was converted to rHDL particle molarity on the basis of the fact that the spherical (A-I)rHDL contained three apoA-I molecules per particle while the spherical (A-II)rHDL contained six apoA-II molecules per particle (Table 3). Results are expressed as mean ± SEM (n = 8); spherical (A-I)rHDL (●) and spherical (A-II)rHDL (○). *Significant difference from spherical (A-I)rHDL by ANOVA, $P < 0.05$. All paired points (i.e., points that had a corresponding point in the opposing data set) were treated as a group and used for the analysis of variance; points that were not paired were excluded from the analysis.

TABLE 3. Size and composition of spherical (A-I) and (A-II)rHDL

rHDL Species	Stokes Diameter	Composition[a]				
		A-I	A-II	PL	CE	UC
	nm	*mol/mol rHDL*				
Spherical (A-I)	9.4	3.0	—	97.4	76.6	10.8
Spherical (A-II)	9.8	0.1	6.0	90.8	76.2	11.1

The spherical (A-I)rHDL were prepared as outlined in the legend to Table 1. The spherical (A-II)rHDL were prepared by displacing apoA-I from (A-I)rHDL with lipid-free apoA-II as described in Methods. The number of moles of apolipoprotein per mole of rHDL was determined by cross-linking (15). The number of moles of the other constituents, relative to that of apolipoprotein, was determined by compositional analysis; A-I, apoA-I; A-II, apoA-II; PL, phospholipid; CE, cholesteryl ester; UC, unesterified cholesterol.

[a] Values represent the means of triplicate determinations on five independent rHDL preparations.

coidal (A-I)rHDL as inhibitors. *iii*) The particle size of spherical (A-I)rHDL has no influence on the inhibition. *iv*) Spherical rHDL that contain apoA-I are superior as inhibitors to those containing apoA-II when the rHDL are equated in terms of apolipoprotein molarity, although this superiority is no longer apparent when the preparations are equated in terms of particle molarity. *v*) Replacement of cholesteryl esters with triglyceride in the core of spherical (A-I)rHDL has no effect on the ability of these particles to inhibit VCAM-1 expression.

The best known function of HDL relates to their ability to promote the efflux of cholesterol from cells (22, 23). This cholesterol efflux is promoted by all major subpopulations of native HDL (24–26) as well as by lipid-free apoA-I (27–30) and phospholipid vesicles (31). The observation in the present studies that neither lipid-free apoA-I nor POPC SUVs inhibited VCAM-1 expression suggests that the HDL-mediated inhibition of adhesion molecule expression may involve a mechanism distinct from that which mediates the efflux of cholesterol. The finding that lipid-free apoA-I lacks inhibitory activity has also been made by

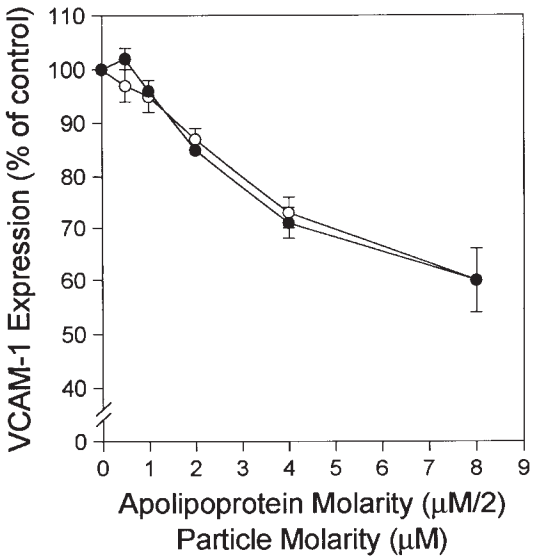

Fig. 5. The effect of the core lipid composition of spherical (A-I) rHDL on the TNF-α induced expression of VCAM-1 by HUVECs. Spherical (A-I)rHDL containing either cholesteryl esters or triglyceride in the core were prepared as outlined in Table 4. HUVECs were pre-incubated with CE or TG core spherical (A-I)rHDL for 16 h before stimulation of the cells with TNF-α. VCAM-1 expression was determined 5 h after stimulation with TNF-α. Results are expressed as mean ± SEM (n = 5). Spherical (A-I)rHDL with a core of cholesteryl esters (●); spherical (A-I)rHDL with a core of triglyceride (○).

TABLE 4. Size and composition of spherical (A-I)rHDL-containing cores of cholesteryl esters or triglyceride

rHDL Species	Stokes Diameter	Composition[a]				
		A-I	PL	CE	UC	TG
	nm	*mol/mol rHDL*				
CE core spherical (A-I)	7.7	2.0	67.4	28.6	5.9	—
TG core spherical (A-I)	7.9	2.0	78.5	0.5	2.2	14.1

The spherical (A-I)rHDL containing a core of cholesteryl esters were prepared as described for the small (A-I)rHDL in the legend to Table 2. The particles with a core of triglyceride were prepared by incubating preparations of large (A-I)rHDL with CETP and Intralipid as described in Methods. The number of moles of apoA-I per mole of rHDL was determined by cross-linking (20). The number of moles of the other constituents, relative to that of apoA-I, was determined by compositional analysis; A-I, apoA-I; PL, phospholipid; CE, cholesteryl ester; UC, unesterified cholesterol; TG, triglyceride.

[a] Values represent the means of triplicate determinations on two rHDL preparations.

Calabresi et al. (11), although these workers found that egg phosphatidylcholine liposomes did have a minor inhibitory effect. In that study however, concentration dependence studies were not performed with phospholipids and it was not stated whether the phospholipid-mediated inhibition was statistically significant.

The fact that discoidal complexes of POPC and apoA-I inhibited VCAM-1 expression, while the individual constituents did not (Fig. 1) may be interpreted in several ways. It is possible that the apolipoprotein is crucial to the effect but only when it is in a conformation that results from its association with phospholipid. Indeed, major structural changes in apoA-I, such as increases in α-helical content, tryptophan exposure, and conformational stability, have been shown to occur when lipid-free apoA-I becomes associated with phospholipid and cholesterol (32–34). It is also possible that the key inhibitory component is the phospholipid which has to be associated with an apolipoprotein in order to be targeted to its site of action. Whether the inhibition requires binding of HDL to a cell surface receptor is unknown. The fact that lipid-free apoA-I binds to putative HDL receptors (35, 36), however, suggests that binding per se may not completely explain the inhibition of VCAM-1 expression, although Sviridov et al. (37) have shown that while lipid-free apoA-I does bind to putative HDL receptors, its association with these receptors is some 70 to 80% lower than that observed with native HDL_3 and discoidal (A-I)rHDL.

The HDL to which endothelial cells are exposed in vivo are predominantly spherical rather than discoidal particles. It was therefore of considerable interest to discover that spherical (A-I)rHDL were superior to discoidal (A-I) rHDL as inhibitors of TNF-α-induced endothelial cell adhesion molecule expression. This result is in marked contrast to HDL-mediated cholesterol efflux which is promoted to a greater extent by discoidal particles than by spherical particles (23).

Spherical and discoidal (A-I)rHDL differ not only in shape and composition (Table 1), but also in several structural respects. For example, the apoA-I in spherical (A-I)rHDL is in a more stable conformation than that in discoidal (A-I)rHDL (16, 38) suggesting that there may be differences in the binding of apoA-I to lipid in these particles. In addition, phospholipid acyl-chain and head group packing is more ordered in spherical (A-I)rHDL than in discoidal (A-I)rHDL (16, 38). There are also differences in the α-helical content of apoA-I in spherical and discoidal (A-I)rHDL and in the microenvironment around the lysine residues on apoA-I in these particles (39, 40). Precisely how these structural differences between apoA-I in spherical and discoidal HDL relate to their relative abilities to inhibit VCAM-1 expression remains to be determined.

It is possible that differences in the inhibitory activity of spherical and discoidal rHDL may reflect differences in surface and core lipid composition. Spherical (A-I)rHDL prepared as described in this study are known to contain sphingomyelin (16), they are also thought to contain different phospholipid species on their surface (specifically phosphatidylethanolamine and phosphatidylcholine species other than POPC), which have transferred from LDL to the spherical rHDL during incubation with LCAT. It will be of interest to investigate whether such differences in phospholipid composition influence the ability of HDL to inhibit VCAM-1 expression.

The effect of apolipoprotein composition on the ability of spherical rHDL to inhibit VCAM-1 expression depended on whether concentrations were equated in terms of apolipoprotein molarity or rHDL particle molarity. When the preparations were equated in terms of apolipoprotein molarity (Fig. 4a), the inhibitory activity of (A-I)rHDL was clearly superior to that of (A-II)rHDL. However, when the rHDL were compared at equivalent particle molarities, this difference in inhibitory activities was no longer apparent (Fig. 4b), suggesting that it is the number of HDL particles rather than their apolipoprotein composition that determines the extent of VCAM-1 inhibition. Apolipoprotein composition has been reported to influence other functions of HDL. Some workers have reported that (A-I)HDL are superior to (A-II)HDL as acceptors of the cholesterol released from cells (25, 41), although others have failed to detect such a difference (26). It has also been observed in studies of transgenic animals that (A-I)HDL are superior to (A-II)HDL as inhibitors of the development of atherosclerosis (42–46).

Changes to the core lipids of spherical (A-I)rHDL had no discernible effect on the ability of the particles to inhibit VCAM-1 expression. Even when virtually all of the cholesteryl esters in the core were replaced by triglyceride, there was no change in the magnitude of inhibition. It would seem, therefore, that HDL core lipid plays no major part in the ability of HDL to inhibit the expression of adhesion molecule. This observation is consistent with an inhibitory mechanism for adhesion molecule expression in which the surface of the HDL particle plays an integral part.

The present studies have been conducted exclusively with HUVECs, although in previous studies from this laboratory we have demonstrated that HDL also inhibit TNF-α-induced VCAM-1 expression in human umbilical arterial

endothelial cells (G. W. Cockerill, unpublished observations). This is in agreement with a recent comparative study by Klein et al. (47) investigating the effect of TNF-α on VCAM-1 expression in human umbilical vein or femoral artery endothelial cells. These investigators concluded that HUVECs do represent a relevant model for studying the regulation of endothelial cell adhesion molecule expression. To this extent it is reasonable to conclude that the results of the present study can be extrapolated to what may occur in arterial endothelial cells.

Furthermore, HDL have been reported previously to inhibit the expression not only of VCAM-1 but also of intercellular adhesion molecule-1 and E-selectin in HUVECs activated by both TNF-α and interleukin-1 (6). It is reasonable, therefore, to extrapolate the results of the present study beyond the specific case of VCAM-1 expression induced by TNF-α. It remains to be determined, however, whether the results reflect what occurs in vivo.

In conclusion, these studies were designed to investigate whether the shape, size, and composition of HDL influence their ability to inhibit the TNF-α-induced expression of VCAM-1 in HUVECs. Spherical rHDL were clearly superior to discoidal particles. However, when the various preparations of spherical rHDL were compared at equivalent particle molarities, it was apparent that the extent of inhibition was unaffected by differences in particle size, by replacing apoA-I with apoA-II or by replacing most of the cholesteryl esters in the particle core with triglyceride. Studies are now underway to determine whether varying the phospholipid composition has an effect on this potentially anti-inflammatory property of HDL.

Appreciation is given to Mrs. Jenny Drew for her expert technical assistance in preparing HUVECs for these studies. Thanks are also given to Mr. Richard Bright for preparing LCAT and CETP and Dr. Dale T. Ashby for preparing HDL_3. This work was supported by grants from the National Health and Medical Research Council and the National Heart Foundation of Australia.

Manuscript received 7 July 1998 and in revised form 22 October 1998.

REFERENCES

1. Gordon, D. J., and B. M. Rifkind. 1989. High-density lipoprotein: The clinical implications of recent studies. *N. Engl. J. Med.* **321:** 1311–1316.
2. Bruce, C., and A. R. Tall. 1995. Cholesteryl ester transfer protein, reverse cholesterol transport, and atherosclerosis. *Curr. Opin. Lipidol.* **6:** 306–311.
3. Quintao, E. C. R. 1995. Is reverse cholesterol transport a misnomer for suggesting its role in the prevention of atheroma formation? *Atherosclerosis.* **116:** 1–14.
4. Fielding, C. J., and P. E. Fielding. 1995. Molecular physiology of reverse cholesterol transport. *J. Lipid Res.* **36:** 211–228.
5. Mackness, M. I., and P. N. Durrington. 1995. HDL, its enzymes and its potential to influence lipid peroxidation. *Atherosclerosis.* **115:** 243–253.
6. Cockerill, G. W., K-A. Rye, J. R. Gamble, M. A. Vadas, and P. J. Barter. 1995. High-density lipoproteins inhibit cytokine-induced expression of endothelial cell adhesion molecules. *Arterioscler. Thromb. Vasc. Biol.* **15:** 1987–1994.
7. Ashby, D. T., K-A. Rye, M. A. Clay, M. A. Vadas, J. R. Gamble, and P. J. Barter. 1998. Factors influencing the ability of HDL to inhibit expression of vascular cell adhesion molecule-1 in endothelial cells. *Arterioscler. Thromb. Vasc. Biol.* **18:** 1450–1455.
8. Li, H., M. I. Cybulsky, M. A. Gimbrone, and P. Libby. 1993. An atherogenic diet rapidly induces VCAM-1, a cytokine-regulable mononuclear leukocyte adhesion molecule, in rabbit aortic endothelium. *Arterioscler. Thromb.* **13:** 197–204.
9. Davies, M. J., J. L. Gordon, A. J. H. Gearing, R. Pigott, N. Woolf, D. Katz, and A. Kyriakopoulos. 1993. The expression of the adhesion molecules ICAM-1, VCAM-1, PECAM, and E-selectin in human atherosclerosis. *J. Pathol.* **171:** 223–229.
10. Peter, K., P. Nawroth, C. Condradt, T. Nordt, T. Weiss, M. Boehme, A. Wunsch, J. Allenberg, W. Kubler, and C. Bode. 1997. Circulating vascular cell adhesion molecule-1 correlates with the extent of human atherosclerosis in contrast to circulating intracellular adhesion molecule-1, E-selectin, P-selectin, and thrombomodulin. *Arterioscler. Thromb. Vasc. Biol.* **17:** 505–512.
11. Calabresi, L., G. Franceschini, C. R. Sirtori, A. De Palma, M. Saresella, P. Ferrante, and D. Taramelli. 1997. Inhibition of VCAM-1 expression in endothelial cells by reconstituted high density lipoproteins. *Biochem. Biophys. Res. Commun.* **238:** 61–65.
12. Moudry, R., M. O. Spycher, and J. E. Doran. 1997. Reconstituted high density lipoprotein modulates adherence of polymorphonuclear leukocytes to human endothelial cells. *Shock.* **7:** 175–181.
13. Osborne, J. C. 1986. Delipidation of plasma lipoproteins. *Methods Enzymol.* **128:** 213–222.
14. Rye, K-A., K. H. Garrety, and P. J. Barter. 1993. Preparation and characterization of spheroidal, reconstituted high-density lipoproteins with apolipoprotein A-I only or with apolipoprotein A-I and A-II. *Biochim. Biophys. Acta.* **1167:** 316–325.
15. Rye, K-A., N. J. Hime, and P. J. Barter. 1997. Evidence that cholesteryl ester transfer protein-mediated reductions in reconstituted high density lipoprotein size involve particle fusion. *J. Biol. Chem.* **272:** 3953–3960.
16. Rye, K-A., N. J. Hime, and P. J. Barter. 1996. The influence of sphingomyelin on the structure and function of reconstituted high density lipoproteins. *J. Biol. Chem.* **271:** 4243–4250.
17. Jonas, A. 1986. Reconstitution of high-density lipoproteins. *Methods Enzymol.* **128:** 553–582.
18. Matz, C. E., and A. Jonas. 1982. Micellar complexes of human apolipoprotein A-I with phosphatidylcholines and cholesterol prepared from cholate-lipid dispersions. *J. Biol. Chem.* **257:** 4535–4540.
19. Rye, K-A., and P. J. Barter. 1994. The influence of apolipoproteins on the structure and function of spheroidal, reconstituted high density lipoproteins. *J. Biol. Chem.* **269:** 10298–10303.
20. Rye, K-A., N. J. Hime, and P. J. Barter. 1995. The influence of cholesteryl ester transfer protein on the composition, size, and structure of spherical, reconstituted high density lipoproteins. *J. Biol. Chem.* **270:** 189–196.
21. Wall, R. T. 1978. Factors influencing endothelial cell proliferation in vitro. *J. Cell Physiol.* **96:** 203–213.
22. Oram, J. F., and S. Yokoyama. 1996. Apolipoprotein-mediated removal of cellular cholsterol and phospholipids. *J. Lipid Res.* **37:** 2473–2491.
23. Johnson, W. J., F. H. Mahlberg, G. H. Rothblat, and M. C. Phillips. 1991. Cholesterol transport between cells and high-density lipoproteins. *Biochim. Biophys. Acta.* **1085:** 273–298.
24. de la Llera Moya, M., V. Atger, J. L. Paul, N. Fournier, N. Moatti, P. Giral, K. E. Friday, and G. Rothblat. 1994. A cell culture system for screening human serum for ability to promote cellular cholesterol efflux. Relations between serum components and efflux, esterification, and transfer. *Arterioscler. Thromb.* **14:** 1056–1065.
25. Barkia, A., P. Puchois, N. Ghalim, G. Torpier, R. Barbaras, G. Ailhaud, and J. C. Fruchart. 1991. Different role of apolipoprotein A-I-containing particles in cholesterol efflux from adipose cells. *Atherosclerosis.* **87:** 135–146.
26. Oikawa, S., A. J. Mendez, J. F. Oram, E. L. Bierman, and M. C. Cheung. 1993. Effects of high-density lipoprotein particles containing apoA-I with or without apoA-II, on intracellular cholesterol efflux. *Biochim. Biophys. Acta* **1165:** 327–334.
27. Hara, H., and S. Yokoyama. 1991. Interaction of free apolipoproteins with macrophages. Formation of high density lipoprotein-like lipoproteins and reduction of cellular cholesterol. *J. Biol. Chem.* **266:** 3080–3086.
28. Yancey, P. G., J. K. Bielicki, W. J. Johnson, S. Lund-Katz, M. N. Palgunachari, G. M. Anantharamaiah, J. P. Segrest, M. C. Phillips, and G. H. Rothblat. 1995. Efflux of cellular cholesterol and phospho-

lipid to lipid-free apolipoproteins and class A amphipathic peptides. *Biochemistry.* **34:** 7955–7965.
29. Li, Q., H. Czarnecka, and S. Yokoyama. 1995. Involvement of a cellular surface factor(s) in lipid-free apolipoprotein-mediated cellular cholesterol efflux. *Biochim. Biophys. Acta.* **1259:** 227–234.
30. Sviridov, D., L. E. Pyle, and N. Fidge. 1996. Efflux of cellular cholesterol and phospholipid to apolipoprotein A-I mutants. *J. Biol. Chem.* **271:** 33277–33283.
31. DeLamatre, J., G. Wolfbauer, M. C. Phillips, and G. H. Rothblat. 1986. Role of apolipoproteins in cellular cholesterol efflux. *Biochim. Biophys. Acta.* **875:** 419–428.
32. Davidson, W. S., K. L. Gillotte, S. Lund-Katz, W. J. Johnson, G. Rothblat, and M. C. Phillips. 1995. The effect of high density lipoprotein phospholipid acyl chain composition on the efflux of cellular free cholesterol. *J. Biol. Chem.* **270:** 5882–5890.
33. Jonas, A., K. E. Kezdy, and J. Hefele Wald. 1989. Defined apolipoprotein A-I conformations in reconstituted high density lipoprotein discs. *J. Biol. Chem.* **264:** 4818–4824.
34. Sparks, D. L., W. S. Davidson, S. Lund-Katz, and M. C. Phillips. 1993. Effect of cholesterol on the charge and structure of apolipoprotein A-I in recombinant high density lipoprotein particles. *J. Biol. Chem.* **268:** 23250–23257.
35. Mendez, A. J., G. M. Anantharamaiah, J. P. Segrest, and J. F. Oram. 1994. Synthetic amphipathic helical peptides that mimic apolipoprotein A-I in clearing cellular cholesterol. *J. Clin. Invest.* **94:** 1698–1705.
36. Bond, H. M., G. Morrone, S. Venuta, and K. E. Howell. 1991. Characterization and purification of proteins which bind high-density lipoprotein. A putative cell-surface receptor. *Biochem. J.* **279:** 633–641.
37. Sviridov, D. D., A. Y. Misharin, I. G. Safonova, N. G. Bushmakina, V. S. Repin, and V. N. Smirnov. 1988. Binding of partially reassembled high-density lipoprotein to isolated human small intestine epithelial cells. Effect of lipid composition. *Biochim. Biophys. Acta.* **963:** 119–125.
38. Jonas, A., J. H. Wald, K. L. Harms Toohill, E. S. Krul, and K. E. Kezdy. 1990. Apolipoprotein A-I structure and lipid properties in homogeneous, reconstituted spherical and discoidal high density lipoproteins. *J. Biol. Chem.* **265:** 22123–22129.
39. Sparks, D. L., S. Lund-Katz, and M. C. Phillips. 1992. The charge and structural stability of apolipoprotein A-I in discoidal and spherical recombinant high density lipoprotein particles. *J. Biol. Chem.* **267:** 25839–25847.
40. Sparks, D. L., M. C. Phillips, and S. Lund-Katz. 1992. The conformation of apolipoprotein A-I in discoidal and spherical recombinant high density lipoprotein particles. *J. Biol. Chem.* **267:** 25830–25838.
41. Fruchart, J. C., C. De Geteire, B. Delfly, and G. R. Castro. 1994. Apolipoprotein A-I-containing particles and reverse cholesterol transport: evidence for connection between cholesterol efflux and atherosclerosis risk. *Atherosclerosis.* **110:** S35–S39.
42. Duverger, N., H. Kruth, F. Emmanuel, J-M. Caillaud, C. Viglietta, G. Castro, A. Taileux, C. Fievert, J. C. Fruchart, L. M. Houdebine, and P. Denefle. 1996. Inhibition of atherosclerosis development in cholesterol-fed human apolipoprotein A-I-transgenic rabbits. *Circulation.* **94:** 713–717.
43. Warden, C. H., C. C. Hedrick, J. H. Qiao, L. W. Castellani, and A. L. Lusis. 1993. Atherosclerosis in transgenic mice overexpressing apolipoprotein A-II. *Science.* **261:** 469–472.
44. Pastzy, C., N. Maeda, J. Verstuyft, and E. M. Rubin. 1994. Apolipoprotein A-I transgene corrects apolipoprotein E deficiency-induced atherosclerosis in mice. *J. Clin. Invest.* **94:** 899–903.
45. Rubin, E. M., R. M. Krauss, E. A. Spangler, J. G. Verstuyft, and S. M. Clift. 1991. Inhibition of early atherogenesis in transgenic mice by human apolipoprotein A-I. *Nature.* **353:** 265–267.
46. Schultz, J. R., J. G. Verstuyft, E. L. Gong, A. V. Nichols, and E. M. Rubin. 1993. Protein composition determines the anti-atherogenic properties of HDL in transgenic mice. *Nature.* **365:** 762–764.
47. Klein, C., H. Kohler, F. Bittinger, M. Wagner, I. Hermanns, K. Grant, J. C. Lewis, and C. J. Kirkpatrick. 1994. Comparative studies on vascular endothelium in vitro. I. Cytokine effects on the expression of adhesion molecules by human umbilical vein, saphenous vein and femoral artery endothelial cells. *Pathobiology.* **62:** 199–208.