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# Inhibition of VCAM-1 Expression in Endothelial Cells by Reconstituted High Density Lipoproteins

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**Plasma-derived high density lipoproteins (HDL) were found to inhibit cytokine-induced expression of endothelial cell adhesion molecules. Here we used apolipoprotein-specific reconstituted HDL (rHDL) made with phosphatidylcholine (PC) and three different apolipoproteins to identify the HDL components involved in this effect. rHDL containing apolipoprotein A-I (apoA-I), the disulfide-linked form of the apoA-I$_{Milano}$ variant, or apoA-II, were all effective in inhibiting the expression of vascular cell adhesion molecule-1 (VCAM-1) and intercellular adhesion molecule-1 in TNF$\alpha$- or LPS-stimulated HUVEC. The inhibition was concentration dependent in the range of 0.1–1.0 mg/ml (protein). PC liposomes slightly depressed TNF$\alpha$-induced VCAM-1 expression (16% vs 43–50% for the various rHDL), whereas the lipid-free apolipoproteins had no effect. The protein component of HDL is involved in the inhibition of VCAM-1 expression in HUVEC through a rather unspecific mechanism, as three apolipoproteins with remarkably different primary structure display very similar activity.** © 1997 Academic Press

A number of cross-sectional and prospective studies have shown a strong inverse correlation between the plasma concentration of high density lipoprotein (HDL) cholesterol and the incidence of coronary heart disease (1). Further direct evidence for the protective role of HDL against atherosclerosis comes from studies in transgenic mice and in HDL-injected animals. The overexpression in mice of human apolipoprotein A-I (apoA-I), the major protein component of HDL, raises plasma HDL levels and confers protection against diet- or gene-induced atherosclerosis (2,3). The intravenous administration of native or synthetic HDL to cholesterol-fed rabbits inhibits the formation of arterial lesions in various models of vascular injury (4-6).

The protective role of HDL is believed to be mostly due to their function in reverse cholesterol transport, the process by which excess cholesterol in the arterial wall is carried to the liver for excretion (7). However, studies in HDL-treated rabbits argue for other potential mechanisms, as the injection of synthetic HDL caused a 50-70% inhibition of intimal thickening induced by vascular injury, without changing aortic cholesterol and the macrophage content of the intima (5,6). Since synthetic HDL were effective in inhibiting intimal thickening only when administered before injury, a preventive effect on early events in lesion formation can be hypothesized.

The onset of a vascular lesion involves adhesion of blood leukocytes to the dysfunctional endothelium and subsequent transmigration of these cells into the intimal tissue (8). These processes appear to depend on the coordinated expression and activation of a complex network of cellular adhesion molecules and their cognate ligands in response to a wide variety of stimuli (9). The endothelial cell adhesion molecules (CAMs), vascular cell adhesion molecule-1 (VCAM-1, or CD106), and intercellular adhesion molecule-1 (ICAM-1 or CD54), are expressed at early stages of atherogenesis (9,10), and may contribute to the progression of the disease (11). In a recent study, plasma-derived HDL have been shown to modulate CAMs expression on cultured endothelial cells (12), an effect which might well contribute to the protection from atherosclerosis observed *in vivo*.

Plasma HDL are heterogeneous in size, lipid and apolipoprotein composition (13). There is evidence that HDL subpopulations with distinct apolipoprotein content differ in their antiatherogenic properties, possibly because of different functions in reverse cholesterol transport (14). Most of the HDL activities in this complex process, as promotion of cell cholesterol efflux and LCAT-mediated cholesterol esterification, can be mimicked by well defined, apolipoprotein-specific reconstituted HDL (rHDL), which proved to be a valuable tool in identifying apolipoprotein requirements for specific



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HDL functions (15,16). To investigate whether apolipoprotein composition might also affect CAMs modulation by HDL, we examined the ability of apolipoprotein-specific rHDL to regulate CAMs expression on cultured endothelial cells.

## MATERIALS AND METHODS

*Cell culture.* Human umbilical vein endothelial cells (HUVEC) were isolated from umbilical cord veins as described by Jaffe et al (17) and cultured on 5% gelatin-coated tissue culture plates (Costar) at 37°C in 5% $CO_2$. Cells were maintained in medium 199 (HyClone Europe) supplemented with 5% fetal calf serum (HyClone), endothelial cell growth factor (50 $\mu$g/ml) and heparin (100 $\mu$g /ml). More than 90% of HUVEC stained positive with an anti-human von Willebrand factor antibody. Cells were used between the 2nd and 6th *in vitro* passage. Confluent HUVEC were incubated for 18 hours in the presence of different concentrations of rHDL; recombinant TNF$\alpha$ (1000 U/ml) or LPS (1 $\mu$g/ml) were then added for an additional 4 hours and the cell-surface expression of VCAM-1, ICAM-1 and platelet endothelial cell adhesion molecule-1 (PECAM-1 or CD31) was measured. Cell morphology and viability, determined by lactate dehydrogenase activity and trypan blue exclusion, were found to be unchanged under all the conditions used here.

*Flow cytometry.* HUVEC were harvested by mild trypsinization, washed in PBS and incubated for 30 min at 4°C with anti-CD54 (ICAM-1) or anti-CD106 (VCAM-1) monoclonal antibodies (Cymbus Bioscence). Cells were then washed with cold PBS and incubated for 30 min at 4°C with FITC-conjugated goat anti-mouse IgG (Sigma). After washing, cells were fixed with 1% paraformaldheyde and membrane antigen expression was measured by fluorescence activated cell sorting (FACS) with a Coulter EPICS XL cytofluorograph (Coulter Electronics Inc). Each sample counted $1 \times 10^4$ cells. Control cells were incubated with FITC-conjugated isotype-matched, nonrelevant antibodies (Serotec).

*Enzyme linked immunoadsorbent assay (ELISA).* HUVEC monolayers were washed four times with PBS and fixed with 0.1% glutaraldheyde in PBS for 5′ at room temperature. Plates were incubated at 37°C for two hours with 2% skim milk in PBS and for additional 60 min with anti-CD54 or anti-CD31 monoclonal antibodies (Cymbus Bioscience). After two washings with PBS, peroxidase-conjugated, goat anti-mouse IgG (Sigma) were added for 90 min at 37°C and peroxidase activity was assessed using $H_2O_2$-TMB detection system. Optical reading at 450 nm was performed with a microplate spectrophotometer (Molecular Devices).

*Preparation of rHDL.* Apolipoproteins A-I and A-II were purified from human plasma, as previously described (18,19). The disulfide-linked form of the apoA-$I_{Milano}$ variant (A-$I_M$/A-$I_M$) was expressed in *E. coli* and purified by conventional chromatographic procedures (20). Discoidal rHDL containing egg-yolk phosphatidylcholine (EPC) and either apoA-I, A-$I_M$/A-$I_M$, or apoA-II were prepared by the cholate dialysis technique (21), using an EPC:protein weight ratio of 2.5:1. All protein was incorporated into stable rHDL with no lipid-free apolipoprotein left in the final preparations. The size distribution of rHDL was examined by nondenaturing polyacrylamide gradient gel electrophoresis on 8-25% polyacrylamide gels (Pharmacia Biotech) using the Pharmacia Phast System, the 2400 Gelscan XL software, and thyroglobulin (17.0 nm), apoferritin (12.2 nm), lactate dehydrogenase (8.2 nm) and albumin (7.1 nm), as calibration proteins (22). rHDL containing apoA-I or apoA-II consisted of a single population of particles, with a mean diameter of 9.6 nm; rHDL containing A-$I_M$/A-$I_M$ included two major populations of particles with a diameter of 8.2 and 12.5 nm. EPC liposomes were prepared by the same procedure used for rHDL, omitting the protein in the starting mixture. Low density lipoprotein (LDL) were isolated from fasting human plasma by salt density ultracentrifugation (23). Phospholipid and protein concentrations were measured as described in (16).

## RESULTS

Very little expression of VCAM-1 was detected in non stimulated cells; as expected, the exposure of HUVEC for 4 hours to TNF$\alpha$ resulted in a five- to seven-fold induction of the cell-surface expression of VCAM-1 (Fig. 1). rHDL did not affect VCAM-1 expression in non stimulated cells. To investigate the ability of the various rHDL to inhibit TNF$\alpha$-induced expression of VCAM-1, HUVEC monolayers were incubated for 18 hours with rHDL containing apoA-I, A-$I_M$/A-$I_M$, or apoA-II, at physiological concentrations (1 mg/ml of protein), before the addition of TNF$\alpha$. All rHDL effectively inhibited the TNF$\alpha$-induced VCAM-1 expression (Fig. 1), with only minor differences among the various rHDL. Inhibition was not donor or preparation specific, as comparable results were obtained using HUVEC from various donors and different batches of rHDL. In seven different experiments, rHDL containing apoA-I, A-$I_M$/A-$I_M$, or apoA-II inhibited VCAM-1 expression by 50.4±14.8%, 44.8±13.8%, and 42.7±13.7%, respectively. EPC liposomes, when added at the same EPC concentration as rHDL, slightly inhibited TNF$\alpha$-induced VCAM-1 expression (16.1±9.0%); lipid-free apoA-I (1 mg/ml) or native LDL (1 mg/ml of protein) had no effect. Similar results were obtained when HUVEC were stimulated with LPS (data not shown). The inhibition of TNF$\alpha$-induced VCAM-1 expression by rHDL was concentration-dependent in the range of 0.1-1.0 mg/ml (protein) with all rHDL preparations. FACS plots of a representative experiment with A-$I_M$/A-$I_M$-containing rHDL are shown in Fig. 2.

ICAM-1 was expressed at low levels in naive, non-stimulated cells, and the addition of TNF$\alpha$ or LPS enhanced ICAM-1 expression by two- to three-fold, as assessed by FACS analysis. ApoA-I- and A-$I_M$/A-$I_M$-containing rHDL (1 mg/ml of protein) depressed the TNF$\alpha$-induced enhanced expression of ICAM-1 by 21.6±8.0% and 18.7±8.4%, respectively.

In some experiments, the ability of the various rHDL to modulate the expression of PECAM-1 in TNF$\alpha$-stimulated HUVEC was assayed by ELISA. PECAM-1 was constitutively expressed in HUVEC, and TNF$\alpha$ did not affect expression. Pretreatment of cells with the various rHDL at 1 mg/ml (protein) did not change PECAM-1 expression, despite a significant inhibition of VCAM-1 expression in the same cultures (Fig. 3).

## DISCUSSION

HDL are believed to protect against atherosclerosis mainly by acting as the vehicle of cholesterol in reverse cholesterol transport (7). However, animal experiments with synthetic HDL suggest that these lipoproteins

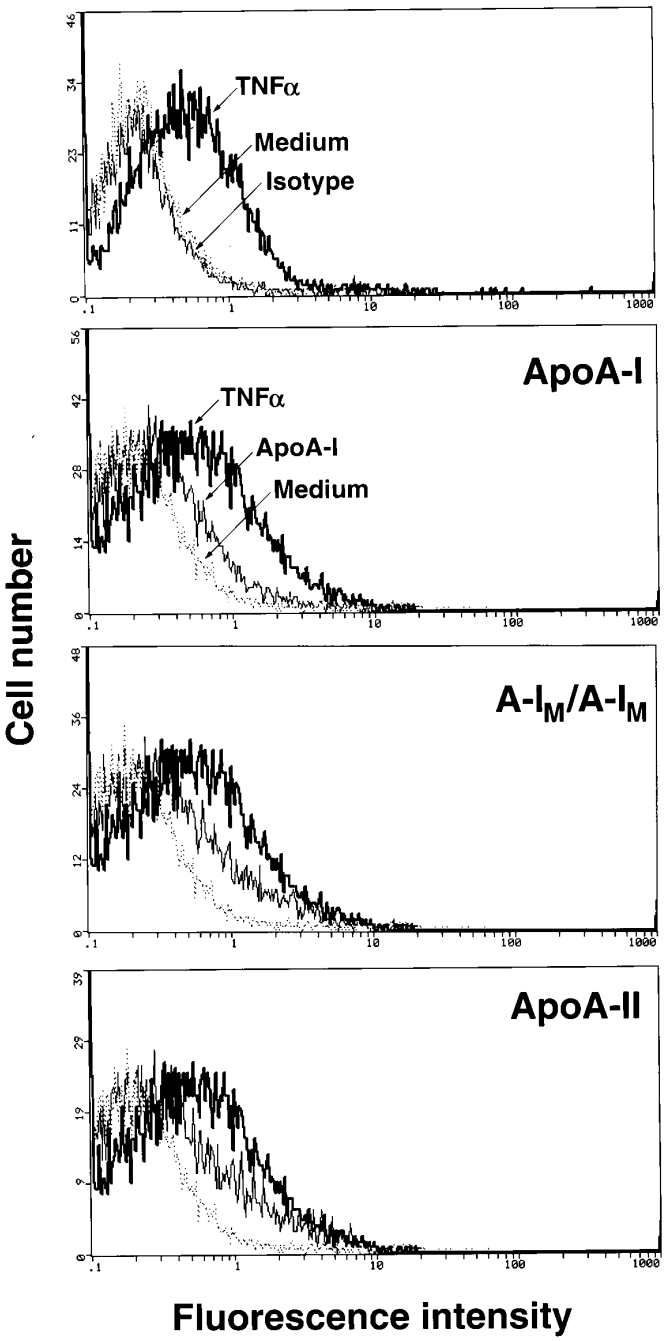


**FIG. 1.** Effect of apolipoprotein-specific rHDL on VCAM-1 induction by TNF$\alpha$. HUVEC were pretreated with rHDL (1 mg/ml of protein) containing apoA-I, A-I$_M$/A-I$_M$, or apoA-II for 18 hours, stimulated with TNF$\alpha$ (1000 U/ml) for 4 hours, stained for VCAM-1 or isotype control, and analyzed by FACS. Shown are original histograms representative of seven experiments with cells from various donors and different rHDL preparations.

may affect atherogenesis at early stages in lesion formation, through mechanisms which are independent of their function in reverse cholesterol transport (5,6), and possibly related to ancillary activities on systems involved in atherogenesis (24). In cocultures of human aortic wall cells, native HDL inhibited LDL-induced binding of monocytes to target endothelial cells, and the subsequent transmigration and localization in the subendothelial space (25). More recently, HDL have been shown to inhibit cytokine-induced CAMs gene transcription in stimulated HUVEC (12). The present study extends these previous observations in showing that the protein component of HDL is involved in the inhibition of VCAM-1 and ICAM-1 cell-surface expression in HUVEC, and that the mechanism responsible for this effect is rather unspecific, as three apolipoproteins with remarkably different primary structure display very similar activity.

CAMs mRNA levels were not measured in the present study; however, rHDL modulation of VCAM-1 and ICAM-1 expression is likely to occur at the transcriptional level, as previously shown for plasma-derived HDL in similarly TNF$\alpha$-stimulated HUVEC (12). The constitutive expression of PECAM-1, which is not sensitive to cytokine-mediated transcriptional regulation (26), was not affected by rHDL, further indicating that they are acting to suppress the synthesis of VCAM-1 and ICAM-1. rHDL containing apoA-I, apoA-II and a disulfide-linked form of the A-I$_{Milano}$ variant all blunted the cytokine-induced expression of VCAM-1 and ICAM-1, suggesting a common mechanism of action.

The transcriptional regulation of CAMs expression in endothelial cells has been studied quite extensively and is likely to involve the activation of the inducible factor NF-$\kappa$B and the assembly of unique transcription activation complexes (27). In the inactive state NF-$\kappa$B is present in the cytosol bound to an inhibitory protein, I$\kappa$B; activation of NF-$\kappa$B by a multitude of stimuli requires the release of the inhibitor, and translocation of NF-$\kappa$B to the nucleus, where it modulates gene transcription (28).

How HDL inhibit cytokine-induced VCAM-1 expression is unclear. An attractive hypothesis is that HDL act as antioxidants. Indeed, native and reconstituted HDL bind lipid peroxides (29) and prevent most of the biological activities of oxidized LDL (30-32). Regulation of VCAM-1 gene expression is coupled to oxidative stress through specific reduction-oxidation sensitive activation of NF-$\kappa$B (33). Cytokine-induced expression of VCAM-1 is enhanced by oxidized LDL (34-36) and inhibited by antioxidants (36-38). HDL may function to control redox-sensitive VCAM-1 gene expression, by scavenging some prooxidant compounds or peroxides generated during HUVEC stimulation (39). Such a mechanism would be consistent with the inhibitory effect of EPC liposomes, which also bind lipid peroxides, on VCAM-1 expression. The much higher activity of rHDL than liposomes suggests that an adequate packing of phospholipids is required for maximal inhibition of VCAM-1 expression. This mechanism would also account for the poor specificity of rHDL containing apoli-

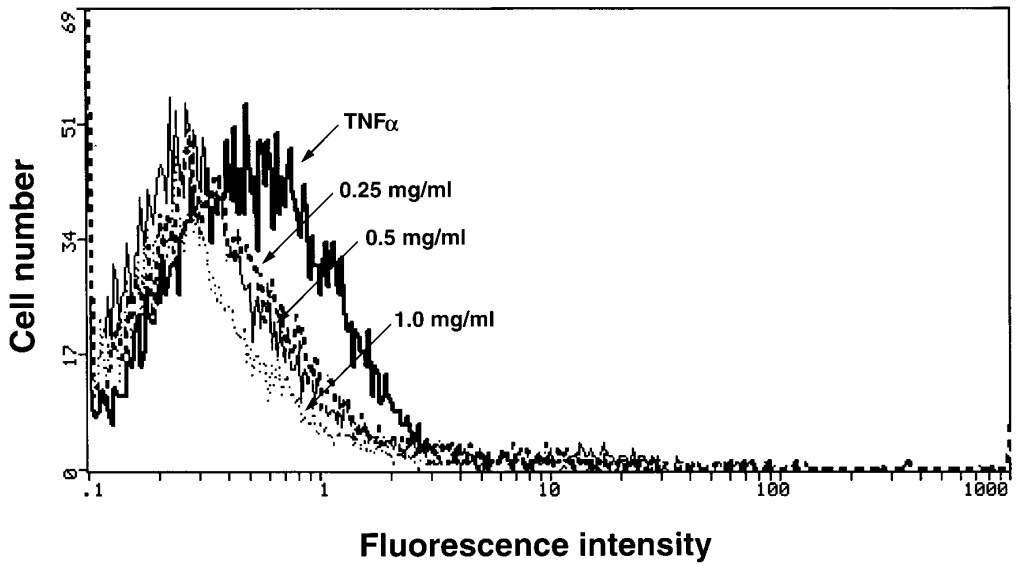


**FIG. 2.** Concentration-dependent inhibition by A-$I_M$/A-$I_M$-containing rHDL of TNF$\alpha$-induced cell-surface expression of VCAM-1. The experimental conditions are the same as for Fig. 1.

poproteins with different primary structure, but all able to generate stable lipid-protein complexes, and for the inability of lipid-free apolipoproteins to modulate VCAM-1 expression.

The present observations provide clear evidence that synthetic HDL containing various apolipoproteins can specifically modulate the expression of biologically active proteins elicited by an inflammatory stimulus. Thus, it is conceivable that *in vivo* one function of HDL is to counteract inflammatory signals involved in atherogenesis. VCAM-1 binds circulating monocytes and may participate in the recruitment of these chronic inflammatory cells from the bloodstream to sites of tissue injury. VCAM-1 expression has been indeed found in aortic endothelial cells overlying foam-cell-rich lesions (10), where it may enhance recruitment of mononuclear cells into the arterial wall, and in the rabbit aorta after vascular injury (40-42), where it may contribute to the chronic phase of intimal proliferation. Therefore, the ability of HDL to inhibit cytokine-induced expression of adhesion molecules may contribute to the protective effect of native HDL against atherosclerosis and may provide an explanation for the reported preventive therapeutic activity of synthetic HDL on lesion development in various animal models of vascular injury.

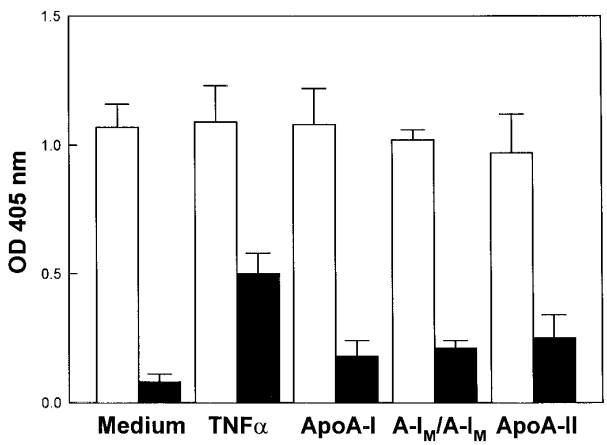


**FIG. 3.** Effect of apolipoprotein-specific rHDL on PECAM-1 (open bars) and VCAM-1 (filled bars) expression. HUVEC were pretreated with rHDL (1 mg/ml of protein) containing apoA-I, A-$I_M$/A-$I_M$, or apoA-II for 18 hours, stimulated with TNF$\alpha$ (1000 U/ml) for 4 hours, stained for PECAM-1 or VCAM-1, and analyzed by ELISA. Results are expressed as mean OD±SD from a representative experiment run in triplicate.

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