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Normal Vascular Function Despite Low Levels of High-Density Lipoprotein Cholesterol in Carriers of the Apolipoprotein A-I$_{Milano}$ Mutant

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Background—Carriers of the apolipoprotein A-I$_{Milano}$ (apoA-I$_M$) mutant have very low plasma high-density lipoprotein cholesterol (HDL-C) levels but do not show any history of premature cardiovascular disease or any evidence of preclinical vascular disease. HDL is believed to prevent the development of vascular dysfunction, which may well contribute to HDL-mediated atheroprotection. Whether the low HDL level of apoA-I$_M$ carriers is associated with impaired vascular function is presently unknown.

Methods and Results—The vascular response to reactive hyperemia, assessed by measuring postischemic increase in forearm arterial compliance, and the plasma concentration of soluble cell adhesion molecules were evaluated in 21 adult apoA-I$_M$ carriers, 21 age- and gender-matched nonaffected relatives (control subjects), and 21 healthy subjects with low HDL-C (low-HDL subjects). The average plasma HDL-C and apoA-I levels of apoA-I$_M$ carriers were remarkably lower than those of control subjects and significantly lower than those of low-HDL subjects. The postischemic increase in forearm arterial compliance in the apoA-I$_M$ carriers was 2-fold greater than in low-HDL subjects and remarkably similar to that of control subjects. Plasma soluble cell adhesion molecule levels were similar in apoA-I$_M$ carriers and control subjects but were greater in low-HDL subjects. When incubated with endothelial cells, HDL isolated from apoA-I$_M$ carriers was more effective than HDL from control and low-HDL subjects in stimulating endothelial nitric oxide synthase expression and activation and in downregulating tumor necrosis factor-α–induced expression of vascular cell adhesion molecule-1.

Conclusions—Despite their very low HDL levels, apoA-I$_M$ carriers do not display typical features of impaired vascular function because of an improved activity of apoA-I$_M$ HDL in maintaining endothelial cell homeostasis. **(*Circulation*. 2007;116:2165-2172.)**

Key Words: lipoproteins ■ apolipoproteins ■ vasodilation ■ nitric oxide ■ cell adhesion molecules

The apolipoprotein A-I$_{Milano}$ (apoA-I$_M$) mutation was first described in 1980 in a family originating from Limone sul Garda in northern Italy.[1] The apoA-I$_M$ mutant differs from wild-type apoA-I by an Arg-to-Cys substitution at position 173 in the primary sequence, which leads to the formation of disulfide-linked homodimers and heterodimers with apoA-II.[2] The apoA-I$_M$ carriers are all heterozygotes for the mutation[3]; they share a proatherogenic lipid profile, characterized by very low plasma high-density lipoprotein cholesterol (HDL-C) levels, normal to somewhat elevated plasma low-density lipoprotein cholesterol levels, and moderate hypertriglyceridemia,[4] but they do not show any history of premature cardiovascular disease[3] or any evidence of vascular disease at a preclinical level.[5] This apparent paradox is explained, at least in part, by an enhanced capacity of serum from apoA-I$_M$ carriers to promote cell cholesterol efflux through the ATP-binding cassette 1 (ABCA1) transporter.[6]

Clinical Perspective p 2172

Vascular dysfunction, characterized by an impaired regulation of vascular tone and by a proinflammatory and proadhesive state of endothelium, is believed to play a key role in the development of atherosclerosis.[7,8] Impaired vascular response has been reported in patients with primary hypoalphalipoproteinemia,[9–11] and significant inverse correlations have repeatedly been found between the plasma HDL-C level and either direct[11,12] or indirect[13] measures of vascular dysfunction. Even more strikingly, a low plasma HDL-C concentration is an independent predictor of impaired peripheral vasodilation in healthy individuals and in hyperlipidemic,

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diabetic, and coronary patients,[14–17] and the elevation of the plasma HDL concentration in patients with low HDL-C levels by either drug treatment or infusion of synthetic HDL leads to a significant improvement of vascular function.[10,18] Together, these findings argue for a protective effect of HDL against the development of vascular dysfunction, which may well contribute, together with the known HDL function in cell cholesterol removal and transport, to HDL-mediated prevention of cardiovascular diseases. Whether the low HDL-C level of apoA-I_M carriers is associated with impaired vascular function is presently unknown. The present study was thus undertaken to compare vascular function in apoA-I_M carriers and in control subjects with either normal or low HDL-C and to evaluate the ability of HDL isolated from carriers and control subjects to maintain endothelial homeostasis in cultured cells.

Methods

Subjects

All apoA-I_M carriers living in Limone sul Garda, Italy, who were between 20 and 70 years of age were invited to be included in a biochemical and cardiovascular checkup study; of the 25 eligible subjects, 21 agreed to participate. An equal number of subjects of the same gender and age were selected among nonaffected relatives (control subjects) and blood donors with low HDL-C (<10th percentile for age- and gender-matched Italian subjects) attending the Servizio Immunoematologico Trasfusionale of the Niguarda Hospital in Milano (low-HDL group).[5] All subjects provided informed consent.

Plasma Lipids and Apolipoproteins

After fasting, blood was collected into tubes containing Na_2-EDTA (final concentration 1 mg/mL); plasma was prepared by low-speed centrifugation, and aliquots were immediately frozen at −80°C. Plasma lipid and apolipoprotein levels were measured as described previously.[5]

Forearm Arterial Compliance

The forearm arterial compliance (FAC) of the nondominant arm was measured at rest and during reactive hyperemia by a previously described plethysmographic method that allowed direct assessment of the nonlinear FAC–blood pressure curve relative to each single cardiac cycle.[12] Diastolic, systolic, and mean blood pressure were recorded continuously from the middle finger of the nondominant arm with a Finapres instrument (Ohmeda, Louisville, Colo). After baseline measurements were taken (average measurement of 16 to 25 beats obtained in 20 seconds), an ischemic occlusion test was performed by upper-arm pressure-cuff inflation 30 mm Hg above the systolic pressure for 3 minutes. After cuff deflation, FAC measurement was performed continuously for an additional 5 minutes. The area under the FAC–blood pressure curve (FAC_{AUC}) was determined at rest (preischemic FAC_{AUC}) and during reactive hyperemia (peak FAC_{AUC}) by calculating the integral between 70 and 130 mm Hg. Postischemic change in FAC_{AUC} was calculated as the difference between preischemic and peak values and expressed as a percentage of the preischemic value. A single observer performed all examinations after patients had rested in a supine position for 15 minutes in a temperature-controlled room. The coefficients of variations for preischemic and peak FAC_{AUC} and postischemic FAC_{AUC} change were <10%.[12]

Plasma Levels of Soluble Cell Adhesion Molecules

Plasma levels of the soluble forms of vascular cell adhesion molecule-1 (VCAM-1), intracellular cell adhesion molecule-1 (ICAM-1), and E-selectin were measured by use of commercially available monoclonal antibody–based ELISA kits (R&D Systems, Minneapolis, Minn). The assays were performed in duplicate for each sample. The operator was blinded as to sample classification.

HDL Preparation

HDLs (d=1.063 to 1.21 g/mL) were isolated from fasting plasma of 6 apoA-I_M carriers (A-I_M HDL), 6 control subjects (control HDL), and 6 low-HDL subjects, all matched for gender and age. Cholesterol and protein concentrations of HDL preparations were determined by standard techniques. HDL preparations were dialyzed against sterilized saline immediately before use.

Cell Cultures and Treatments

Primary cultures of human umbilical vein endothelial cells (HUVECs) were purchased from PromoCell (Heidelberg, Germany) and subcultured for 1 to 3 passages in endothelial growth medium containing 2% fetal calf serum, endothelial cell growth factor/heparin (4 μL/mL), epidermal growth factor (0.1 ng/mL), and fibroblast growth factor (1 ng/mL). To investigate the effects of isolated HDLs on endothelial nitric oxide synthase (eNOS) expression, cells were incubated overnight with increasing concentrations (0.57 mmol/L and 1.14 mmol/L cholesterol) of A-I_M HDL or control HDL. To investigate the effects of HDL on eNOS activation by phosphorylation, cells were harvested after 10 or 20 minutes' incubation with HDL. To investigate the ability of HDL to downregulate cytokine-induced VCAM-1 expression, cells were incubated overnight with increasing concentrations of A-I_M HDL or control HDL (0.28, 0.57, and 1.14 mmol/L cholesterol), washed with PBS to remove HDL, and stimulated with tumor necrosis factor-α (TNF-α; 10 ng/mL) for 4 to 8 hours. At the end of the experiments, cells and conditioned media were immediately frozen at −20°C.

Western Blotting

Cells were harvested in lysis buffer (20 mmol/L Tris, 4% SDS, 20% glycerol containing 1 mmol/L EDTA, 1 mmol/L sodium orthovanadate, 1 mmol/L NaF, 1 μg/mL leupeptin, 1 mmol/L benzamidine, 10 μg/mL soy trypsin inhibitor, 1 mmol/L PMSF, and 0.5 mmol/L DTT, pH 6.8). Cell debris was removed by centrifugation (10 000*g* for 5 minutes), and protein concentration was determined by the microbicinchoninic acid assay. Protein (30 μg) was separated on 10% SDS-PAGE and then transferred on a nitrocellulose membrane. After saturation with 5% nonfat dried milk, membranes were incubated with primary antibodies against total eNOS (BD Biosciences, San Jose, Calif), phosphorylated eNOS (Ser1177, Cell Signaling Technology, Danvers, Mass), or human VCAM-1 (Exalpha Biologicals, Watertown, Mass) and then with horseradish peroxidase–conjugated secondary antibodies (DakoCytomation, Glostrup, Denmark). Bands were visualized by enhanced chemiluminescence (GE Healthcare Biosciences, Uppsala, Sweden). Membranes were then stripped and reprobed with an antibody against β-actin (Sigma-Aldrich Chemie, Steinheim, Germany). Band densities were evaluated with a GS-690 Imaging Densitometer and Multi-Analyst software (Bio-Rad Laboratories, Hercules, Calif).

Reverse-Transcription Polymerase Chain Reaction

Total RNA was extracted from HUVECs with TRIzol reagent (Invitrogen, Carlsbad, Calif). cDNA was prepared by reverse transcription of 1 μg of total RNA with the iScript cDNA synthesis kit (Bio-Rad) and amplified for 25 cycles with iTaq DNA polymerase (Bio-Rad) in a MyCycler (Bio-Rad). The following primers were used: eNOS, sense 5′-GACGCTACGAGGAGTGGAAG-3′, antisense 5′-TAGGCTTTGGGGTTGTCAGG-3′; VCAM-1, sense 5′-GAAGATG-GTCGTGATCCTTG-3′, antisense 5′-ACTTGACTG-TGATC-GGCTTC-3′; and GAPDH, sense 5′-CCACCCATGG-CAAATTCCATGGCA-3′, antisense 5′-TCTAGACGGCAGGTC-AGGTCCACC-3′. Polymerase chain reaction products were separated by electrophoresis on 1% agarose gel, stained with ethidium bromide, visualized by ultraviolet irradiation, and photographed with Polaroid film. Band densities were evaluated with a GS-690 imaging densitometer and Multi-Analyst software (Bio-Rad). VCAM-1 and eNOS band intensities were normalized by their GADPH values.

Table 1. Demographic, Clinical, and Lipid/Lipoprotein Data in ApoA-I_M Carriers, Low-HDL Subjects, and Control Subjects

	ApoA-I_M Carriers	Low-HDL Subjects	Control Subjects
n	21	21	21
Male/female	11/10	11/10	11/10
Age, y	42.2±3.8	43.6±3.6	40.1±3.4
BMI, kg/m^2	25.1±0.6	25.6±0.6	24.1±0.7
SBP, mm Hg	135.2±5.3	124.7±2.5	125.7±4.3
DBP, mm Hg	84.2±2.4	77.5±1.6	79.5±1.3
Smokers, n	7	6	7
Total cholesterol, mg/dL	189.2±10.8	191.4±7.3	202.0±10.3
LDL cholesterol, mg/dL	131.7±7.6	133.5±6.3	127.8±8.3
HDL-C, mg/dL	19.8±2.1*†	27.0±1.4*	49.0±4.4
Triglycerides, mg/dL	186.0±23.4*	141.8±10.7	130.2±14.1
ApoA-I, mg/dL	78.5±6.1*†	96.8±2.9*	131.6±6.2
ApoA-II, mg/dL	17.3±1.0*†	26.0±1.3*	38.7±1.6
ApoB, mg/dL	103.3±7.6	96.9±4.5	86.6±5.1

ApoA-I_M carriers indicates carriers of the apoA-I_M mutant; low-HDL, subjects with low HDL-C selected among blood donors; Controls, non affected relatives from the A-I_M kindred[3]; BMI, body mass index; SBP, systolic blood pressure; and DBP, diastolic blood pressure.

Data are expressed as mean±SEM.

*Significantly different from control subjects.

†Significantly different from low-HDL subjects.

ELISA for VCAM-1

VCAM-1 concentration in conditioned media was evaluated on MaxiSorp plates (Nunc GmbH & Co, Wiesbaden, Germany) with the CytoSets matched-antibody-pairs ELISA kit (BioSource International, Camarillo, Calif), according to the manufacturer's instructions. The assays were performed in duplicate for each sample. The operator was blinded as to sample classification.

Statistical Analyses

Results are expressed as mean±SEM unless otherwise specified. Variables with a skewed distribution were log-transformed before analysis. Differences between groups were evaluated by 1-way ANOVA. ANCOVA was used to adjust for age, gender, body mass index, systolic and diastolic blood pressure, smoking habit, total cholesterol, triglyceride, and apoB differences between groups. Group differences with $P<0.05$ were considered statistically significant.

The authors had full access to the data and take full responsibility for its integrity. All authors have read and agree to the manuscript as written.

Results

Subjects

Demographic, clinical, and lipid/lipoprotein data of apoA-I_M carriers, healthy low HDL-C subjects (low-HDL), and nonaffected family members (control subjects) are given in Table 1. The 3 groups were comparable as to gender, age, body mass index, systolic and diastolic blood pressure, and percentage of smokers. There were no significant differences in total and low-density lipoprotein cholesterol or apoB concentrations among the 3 groups. As expected, apoA-I_M carriers and low-HDL subjects had a significantly lower average HDL-C level than control subjects; accordingly, significant reductions were also detected in apoA-I and apoA-II levels. As previously described,[4] the apoA-I_M carriers displayed a moderate hypertriglyceridemia, whereas low-HDL subjects and control subjects had comparable triglyceride levels.

Forearm Arterial Compliance

Vascular response to reactive hyperemia was assessed in the 3 groups of examined subjects noninvasively by a plethysmographic method.[12] The marked increase in forearm blood flow during the early phases of reactive hyperemia is believed to enhance shear stress, which induces the release of nitric oxide (NO), with consequent vasodilation[19] and increased arterial compliance.[12] Preischemic FAC (FAC_{AUC}) was remarkably similar in the 3 groups of investigated subjects (Table 2). After cuff deflation, FAC_{AUC} increased in all subjects; however, and as expected given our previous finding of a direct correlation between plasma HDL-C and postischemic rise of FAC_{AUC},[12] the increase of FAC_{AUC} in low-HDL subjects was half that of control subjects (Table 2). The postischemic increase of FAC_{AUC} in the apoA-I_M carriers was 2-fold greater than in low-HDL subjects and remarkably similar to that of control subjects (Table 2), which indicates that despite the severe hypoalphalipoproteinemia, the apoA-I_M carriers displayed a normal vascular response to reactive hyperemia. When data were adjusted for age, gender, body mass index, systolic and diastolic blood pressure, smoking habit, total cholesterol, triglycerides, and apoB, the apoA-I_M carriers still had a higher postischemic increase in FAC_{AUC} than low-HDL subjects.

HDL-Mediated eNOS Expression and Activation in Cultured Endothelial Cells

Given the central role of NO production via eNOS in hyperemia-induced vasodilation[20] and the proven ability of isolated HDL to promote eNOS expression and activation in

Table 2. Plethysmographic Data in ApoA-I_M Carriers, Low-HDL Subjects, and Control Subjects

	ApoA-I_M Carriers	Low-HDL Subjects	Control Subjects
Preischemic $FAC_{(AUC)} \times 10^{-2}$	4.9 (3.6–9.2)	6.2 (4.7–8.6)	5.2 (3.9–7.5)
Peak $FAC_{(AUC)} \times 10^{-2}$	10.0 (7.1–20.0)	9.8 (8.5–13.7)	11.0 (8.5–15.0)
Postischemic $FAC_{(AUC)}$ increase, %	110.1 (76.2–172.3)	59.1 (46.0–84.1)*†	107.5 (66.9–137.4)

Abbreviations as in Table 1. Results are expressed as median, with interquartile ranges in parentheses. Unit of $FAC_{(AUC)}$=(mL/100 mL forearm/mm Hg)×mm Hg.

*Significantly different from control subjects.

†Significantly different from apoA-I_M carriers.

vitro,[18,21] we investigated whether the normal vascular response to reactive hyperemia observed in vivo in the apoA-I_M carriers (despite the marked HDL reduction) could be due to an enhanced capacity of A-I_M HDL for eNOS expression/activation. When added to HUVECs overnight, both A-I_M HDL and control HDL caused a significant increase in eNOS mRNA and protein abundance, as demonstrated by reverse-transcription polymerase chain reaction and Western blotting analysis, but stimulation of eNOS expression was greater with A-I_M HDL than with control HDL (Figure 1). HDL isolated from low-HDL subjects was as effective as control HDL in enhancing eNOS protein expression (eNOS/β-actin=0.64±0.03, $P<0.05$ versus A-I_M HDL, $P=0.58$ versus control HDL). The stimulation of eNOS expression by HDL was dose-dependent (Data Supplement Figure I); at the highest concentration tested, A-I_M HDL, control HDL, and HDL from low-HDL subjects enhanced eNOS protein by 2.2±0.2-fold, 1.5±0.1-fold, and 1.4±0.1-fold compared with untreated cells, respectively. When the activities of A-I_M HDL and control HDL were compared in terms of their total protein (Data Supplement Figure I) or apoA-I (data not shown) concentrations, A-I_M HDL was still superior to control HDL. In a separate set of experiments, we evaluated the ability of the various HDL preparations to induce eNOS activation. HUVECs were incubated with increasing concentrations of HDL for 10 and 20 minutes, and eNOS phosphorylation was analyzed by immunoblotting. After 10 minutes of incubation, both A-I_M HDL and control HDL caused a significant and concentration-dependent elevation of the phosphorylated/total eNOS ratio (Figure 2); A-I_M HDL was more effective than control HDL, but the difference did not achieve statistical significance. eNOS activation by HDL declined at 20 minutes, when again, a nonsignificantly greater activity of A-I_M HDL than control HDL was observed (Figure 2). HDL isolated from low-HDL subjects was as effective as control HDL in stimulating eNOS activation (at 0.57 mmol/L cholesterol: phosphorylated/total eNOS=0.45±0.10 at 10 minutes and 0.19±0.06 at 20 minutes, $P=0.83$ and 0.79 versus control HDL, respectively).

Plasma Concentration of Soluble CAMs

Enhanced endothelial expression of CAMs is a typical feature of endothelial dysfunction,[22] and increased plasma levels of soluble CAMs have been proposed as an indirect measure of impaired endothelial function.[23] Plasma levels of the soluble forms of VCAM-1, ICAM-1, and E-selectin in the 3 groups

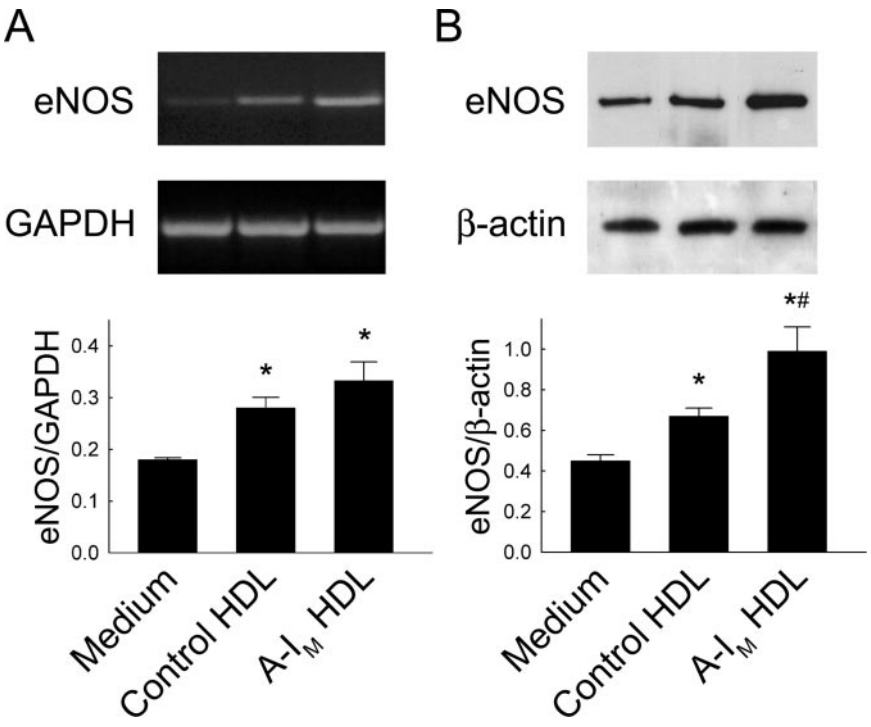

Figure 1. eNOS expression in HDL-treated HUVECs. A, Reverse-transcription polymerase chain reaction analysis for eNOS mRNA levels in untreated cells (medium) and in cells incubated overnight with HDL (1.14 mmol/L cholesterol) isolated from control subjects (control HDL) or apoA-I_M carriers (A-I_M HDL). eNOS mRNA band intensities were normalized by GADPH values. B, Western blotting analysis of eNOS protein levels in untreated cells and in cells incubated overnight with control HDL or A-I_M HDL (1.14 mmol/L cholesterol). eNOS protein band intensities were normalized by β-actin values. Results are expressed as mean±SEM of 3 separate experiments with 3 different preparations of control HDL or A-I_M HDL and 2 batches of cells. *$P<0.05$ vs medium, #$P<0.05$ vs control HDL.

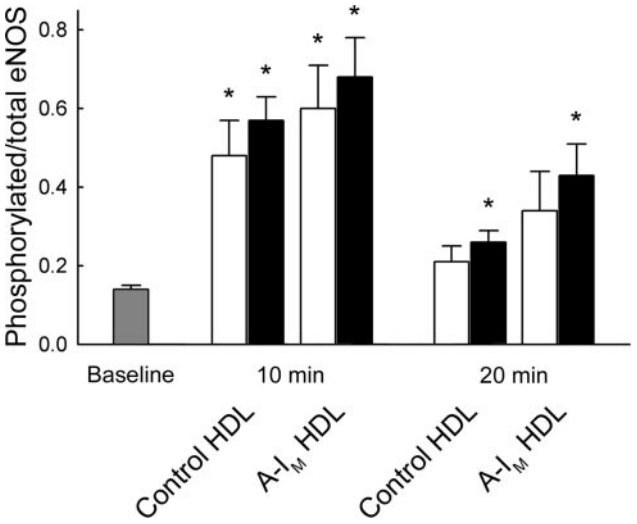

Figure 2. eNOS activation in HDL-treated HUVECs. Cells were incubated with control HDL or HDL from apoA-I_M carriers (A-I_M HDL) at 0.57 mmol/L (open bars) or 1.14 mmol/L (solid bars) cholesterol for 10 or 20 minutes. Western blotting analysis for the phosphorylated and total forms of eNOS was performed, and the phosphorylated/total eNOS ratios were calculated by densitometric analysis. Results are expressed as mean±SEM of 6 separate experiments with 3 different preparations of control HDL or A-I_M HDL and 2 batches of cells. *$P<0.05$ vs baseline.

of investigated subjects are reported in Table 3. Consistent with our previous findings in 2 larger cohorts of subjects with low plasma HDL-C levels,[13] the present low-HDL subjects showed significantly higher plasma levels of soluble VCAM-1, soluble ICAM-1, and soluble E-selectin than control subjects (Table 3). By contrast, no significant difference in plasma soluble CAM levels was found between apoA-I_M carriers and control subjects (Table 3).

HDL-Mediated Inhibition of Endothelial VCAM-1 Expression

HUVECs were preincubated with increasing concentrations of HDL and stimulated with TNF-α before measurement of VCAM-1 concentration in conditioned media by ELISA. Cells were washed with PBS before stimulation to remove HDL and prevent TNF-α sequestration.[24] A-I_M HDL and control HDL both caused a significant and dose-dependent inhibition of VCAM-1 release by stimulated cells (Figure 3), but the inhibition was greater with A-I_M HDL. HDL isolated from low-HDL subjects was as effective as control HDL. At the highest HDL concentration tested, the VCAM-1 content in conditioned media was reduced by 80.2±1.7% with A-I_M HDL, by 50.5±5.8% with control HDL, and by 52.4±3.0% with HDL from low-HDL subjects. When the inhibitory activities of A-I_M HDL and control HDL were compared in terms of their total protein (Figure 3) or apoA-I (data not shown) concentrations, A-I_M HDL was still superior to control HDL. The concentration of VCAM-1 in the conditioned media actually reflects cell expression of VCAM-1, as indicated by Western blotting analysis of cell lysates. VCAM-1 protein was undetectable in unstimulated cells, whereas a massive signal was visible after TNF-α stimulation; preincubation of cells with either A-I_M HDL or control HDL reduced VCAM-1 protein expression compared with stimulated cells (Data Supplement Figure II). As previously shown,[25] inhibition of VCAM-1 expression by HDL occurs at a transcriptional level, as indicated by the HDL-mediated reduction of VCAM-1 mRNA levels in TNF-α–stimulated cells (Figure 4). A-I_M HDL was again more effective than control HDL in downregulating VCAM-1 mRNA levels (Figure 4).

Table 3. Plasma Levels of Soluble CAMs in ApoA-I_M Carriers, Low-HDL Subjects, and Control Subjects

	ApoA-I_M Carriers	Low-HDL Subjects	Control Subjects
sVCAM-1, ng/mL	550.6±32.1	656.3±49.3*	502.6±25.5
sICAM-1, ng/mL	309.8±26.9	335.6±21.5*	267.0±8.9
sE-selectin, ng/mL	52.3±4.3	62.9±4.1*	47.9±3.0

sVCAM-1 indicates soluble VCAM-1; sICAM-1, soluble ICAM-1; and sE-selectin, soluble E-selectin. Other abbreviations as in Table 1.

Data are expressed as mean±SEM.

*Significantly different from control subjects.

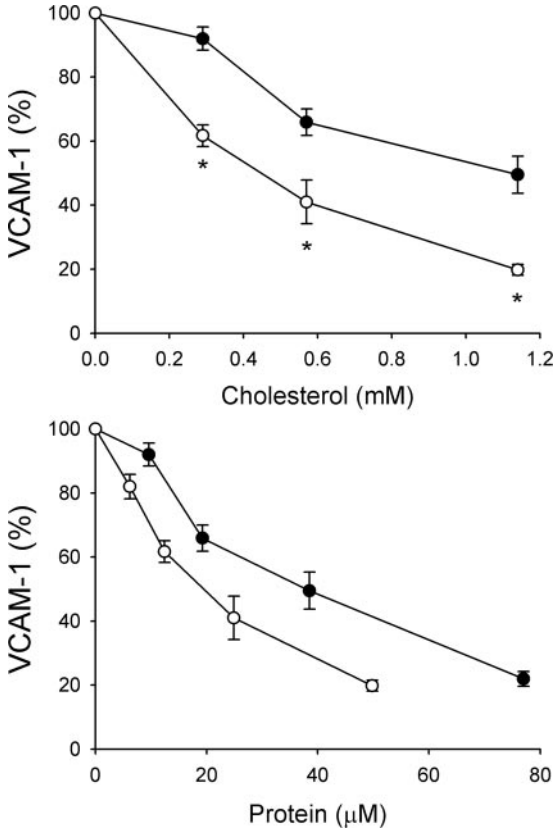

Figure 3. VCAM-1 release in HDL-treated, TNF-α–stimulated HUVECs. Cells were incubated overnight with control HDL (●) or HDL from apoA-I_M carriers (○) before stimulation with TNF-α for 8 hours. Results are expressed as percentage of VCAM-1 concentration in conditioned media of untreated, TNF-α–stimulated HUVECs, mean±SEM of 6 separate experiments with 6 different preparations of control HDL or A-I_M HDL, and 2 batches of cells. *$P<0.05$ vs control HDL.

Discussion

Historical data on the prevalence of cardiovascular disease in the A-I_M kindred[3] and quantitative data on the extent of preclinical atherosclerosis in apoA-I_M carriers[5] demonstrate

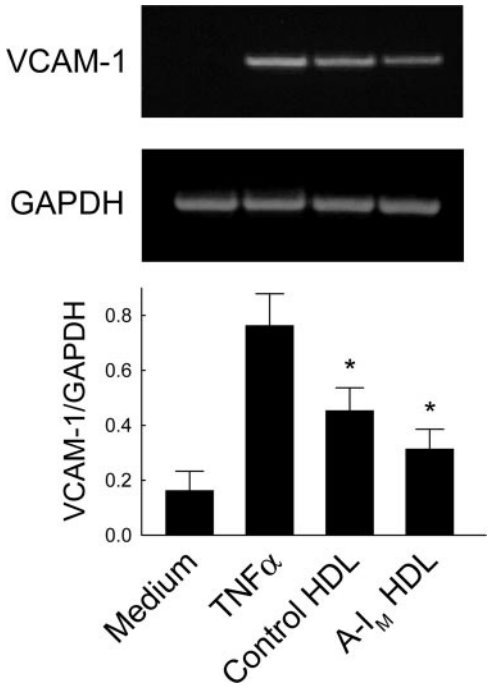

Figure 4. VCAM-1 expression in HDL-treated, TNF-α–stimulated HUVECs. Reverse-transcription polymerase chain reaction analysis for VCAM-1 mRNA levels in untreated, unstimulated cells (medium), in cells stimulated with TNF-α for 4 hours, and in cells incubated overnight with control HDL or HDL from apoA-I_M carriers (A-I_M HDL; 1.14 mmol/L cholesterol) before stimulation with TNF-α. VCAM-1 mRNA band intensities were normalized by GADPH values. Results are expressed as mean±SEM of 6 separate experiments with 6 different preparations of control HDL or A-I_M HDL and 2 batches of cells. *$P<0.05$ vs TNF-α.

that the atherogenic lipid profile and, in particular, the severe hypoalphalipoproteinemia associated with the apoA-I_M mutation do not translate into increased cardiovascular risk. This apparent paradox has been explained by an enhanced capacity of A-I_M HDL to promote cell cholesterol efflux, the first step in reverse cholesterol transport.[6,26] The present investigation provides an additional explanation. Direct and indirect measurements of vascular function demonstrate for the first time that contrary to case subjects with low-HDL who did not have mutations in candidate genes, and despite the severe hypoalphalipoproteinemia, apoA-I_M carriers do not have impaired vascular function. This is likely due to an improved activity of A-I_M HDL to maintain endothelial cell homeostasis, as indicated by the results of in vitro experiments showing that HDL isolated from the plasma of apoA-I_M carriers is more efficient than HDL isolated from control plasma in stimulating eNOS expression and activation and in downregulating cytokine-induced CAM expression.

Vascular function traditionally has been assessed in humans by direct measurement of the endothelial cell response to certain stimuli that trigger the release of NO from the vascular endothelium to mediate vasorelaxation.[27] Reactive hyperemia is another endothelium-dependent stimulus to noninvasively evaluate vascular function.[20] The remarkable increase in forearm blood flow during the early phases of reactive hyperemia enhances shear stress, which in turns induces NO release to enhance compliance during the mid to late phase of reactive hyperemia.[19] The continuous measurement of FAC change during reactive hyperemia, expressed here as postischemic FAC_{AUC} increase, likely reflects the individual's capacity to release NO after a mechanical stimulus.[12] An alternative to functional tests in the assessment of vascular function is the measurement of circulating factors released by damaged and dysfunctional endothelium.[28] Elevated plasma levels of such biomarkers, such as soluble ICAM-1 and soluble VCAM-1, correlate with impaired NO-dependent vasodilation,[29,30] which substantiates their use as a clinical tool for the assessment of endothelial dysfunction.[23] A low plasma HDL-C concentration is an independent predictor of impaired peripheral vasodilation in healthy individuals and in hyperlipidemic, diabetic, and coronary patients.[14–17] The data provided here for apparently healthy low-HDL subjects are fully consistent with these previous findings in showing both a reduced vascular reactivity and an enhanced plasma concentration of biomarkers of endothelial dysfunction, which are likely due to a reduced plasma content of functional HDL. As expected, the average plasma HDL-C of apoA-I_M carriers was remarkably lower than that of control subjects; it was also significantly lower than that of low-HDL subjects recruited for the present study. On the basis of the previously reported direct correlation between plasma HDL-C and postischemic FAC_{AUC} increase,[12] it was expected to be as low as or even lower than that found in low-HDL subjects. The average postischemic FAC_{AUC} increase in the apoA-I_M carriers was instead significantly greater than in low-HDL subjects and remarkably similar to control subjects. Similarly, in view of the inverse correlation between plasma levels of HDL-C and soluble CAMs,[13] the apoA-I_M carriers were expected to have plasma soluble CAM concentrations as high as or even higher than those of low-HDL subjects, but again, this was not true. Therefore, both direct and indirect measures unambiguously show that apoA-I_M carriers do not have impaired vascular function, and thus, they behave differently from other cases with primary hypoalphalipoproteinemia, in whom a remarkable vascular dysfunction has been reported.[9,10,13]

Considerable effort has been expended to understand the mechanisms whereby the apoA-I_M mutation might be linked to cardiovascular protection. Previous in vitro experiments showed an enhanced capacity of A-I_M HDL, compared with HDL isolated from healthy control subjects, to promote cell cholesterol efflux through both the SR-BI (class B, type 1 scavenger receptor) and ABCA1 pathways.[6,26] The present studies demonstrate that A-I_M HDL is also more efficient than control HDL in enhancing endothelial NO bioavailability and in downregulating cytokine-induced endothelial CAM expression. These findings are fully consistent with previous data showing that treatment of isolated rabbit arteries with A-I_M/phospholipid complexes caused a significant attenuation of lysophosphatidylcholine-induced impairment of endothelium-dependent vasodilation, whereas complexes made with wild-type apoA-I, as well as plasma-derived control HDL, produced a nonsignificant attenuation.[31] Together, these observations indicate that the apoA-I_M mutation exerts a gain-of-function effect against the development of cardiovascular disease through at least 2 distinct mechanisms: promotion of

cell cholesterol removal from the arterial wall and maintenance of endothelial cell homeostasis. This conclusion is supported by recent findings in apoA-I/apoE double-knockout mice, in which the expression of apoA-I_M leads to enhanced macrophage cholesterol efflux and reduced arterial monocyte infiltration, thus lowering atherosclerosis burden, compared with mice expressing wild-type apoA-I.[32]

Low plasma HDL-C concentrations are highly prevalent in coronary patients, and genetic variation in HDL candidate genes accounts for a sizable proportion of low HDL-C in the general population.[33] Genetically determined low-HDL states, however, may be associated with an extremely variable atherosclerosis burden[34] and coronary risk.[35] Paradigmatic is the case of molecular defects in the apoA-I gene. Although complete apoA-I deficiency due to chromosomal aberration is definitely associated with premature coronary heart disease,[36] even marked reductions of plasma HDL-C caused by more subtle mutations in the same gene do not necessarily lead to enhanced coronary risk.[34] Carriers of the apoA-I_M mutation, who do not show any structural[5] or functional evidence of vascular disease at the preclinical level, behave clearly distinctly from carriers of other missense apoA-I mutations that lead to hypoalphalipoproteinemia, such as the apoA-I (L178P) mutation, who instead present with accelerated carotid arterial wall thickening and endothelial dysfunction, associated with a severely enhanced cardiovascular risk.[37] This dramatic difference in the clinical phenotype of carriers of 2 missense mutations in the apoA-I gene, both of which lead to remarkably similar plasma HDL-C reductions, illustrates that HDL-C levels per se do not necessarily reflect the atheroprotective potential of HDL and highlights the need for novel tools for cardiovascular risk prediction in individuals with low HDL. Genetic testing aimed at the identification of the molecular defect that causes the low-HDL state does not appear to have predictive properties, except for mutations already known to be associated with either low or high risk. The assessment of HDL in terms of function rather than just levels of cholesterol or apoA-I may provide relevant insight into the atheroprotective capacity of each individual HDL, but simple, reliable, and reproducible assays of HDL function are less likely to be applicable on a large scale or validated against clinical outcomes. Surrogate markers to assess either atherosclerotic burden, such as carotid intima-media thickness, or vascular function, such as those used in the present study, although influenced by a variety of factors other than HDL, have already proved to be strong predictors of future cardiovascular events and appear to be the best tools for the screening of low-HDL individuals and for prioritizing antiatherogenic therapies aimed at increasing plasma HDL levels and function, when these therapies will be made available.

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Disclosures

None.

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CLINICAL PERSPECTIVE

Population studies clearly demonstrate that a low plasma high-density lipoprotein cholesterol (HDL-C) concentration is a strong predictor of future coronary heart disease (CHD). Genetically determined low HDL-C states may, however, be associated with a widely variable CHD risk. Paradigmatic is the case of molecular defects in the apolipoprotein (apo) A-I gene. In the present study, we show that carriers of the apoA-I_M (Arg173C) mutation, who have very low HDL-C levels, do not have vascular dysfunction, likely because of an improved activity of their HDL in maintaining endothelial cell homeostasis. This finding complements previous observations on the lack of preclinical atherosclerosis and premature CHD in the A-I_M kindred. Carriers of other missense apoA-I mutations that lead to low HDL-C, such as the apoA-I (L178P) mutation, instead present with endothelial dysfunction, accelerated carotid arterial wall thickening, and a severely enhanced CHD risk. This dramatic difference illustrates that HDL-C levels per se do not necessarily reflect the atheroprotective potential of HDL and highlights the need for novel tools for CHD risk prediction in individuals with low HDL-C levels. Genetic testing does not appear to have predictive properties, except for already-known mutations. The assessment of HDL in terms of function rather than just HDL-C levels may provide relevant insight into the atheroprotective capacity of each individual HDL, but simple, reliable, and reproducible assays for this purpose are presently unavailable. Surrogate markers to assess atherosclerotic burden or vascular function appear to be the best tools for screening of low-HDL individuals and for prioritizing current and future antiatherogenic therapies.