Elevation of Plasma High-Density Lipoprotein Concentration Reduces Interleukin-1–Induced Expression of E-Selectin in an In Vivo Model of Acute Inflammation

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**Background**—Although there is strong evidence that plasma HDL levels correlate inversely with the incidence of coronary artery disease, the precise mechanism(s) for the protective effect of HDLs remains unclear. We recently showed that HDLs inhibit endothelial cell expression of cytokine-induced leukocyte adhesion molecules in vitro. Our study therefore sought to test the hypothesis that elevating the level of circulating HDLs would inhibit endothelial cell activation in vivo.

**Methods and Results**—We used a porcine model of inflammation previously established in our laboratory, in which the level of vascular endothelial cell expression of E-selectin in interleukin (IL)-1α–induced skin lesions was measured by the uptake of a radiolabeled anti–E-selectin antibody (1.2B6). Porcine plasma HDL levels were elevated by use of a bolus injection of reconstituted discoidal HDL (recHDL). These particles resemble nascent HDL particles in shape and contain apolipoprotein A-I as the sole protein and soybean phosphatidylcholine as the sole phospholipid. We found that recHDLs inhibited the expression of IL-1α–induced E-selectin by porcine aortic endothelial cells in vitro, confirming that the inhibitory effect is conserved with synthetic HDLs and demonstrating that the phenomenon is not restricted to human endothelial cells. In vivo, elevating the circulating level of HDLs ∼2-fold led to significant inhibition of basal and IL-1α–induced E-selectin expression by porcine microvascular endothelial cells.

**Conclusions**—These observations demonstrate the potential anti-inflammatory action of HDLs and provide support for the further investigation of the mechanisms underlying the inhibitory effects of HDLs on endothelial cell activation.

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**Key Words:** inflammation ■ atherosclerosis ■ proteins ■ endothelium ■ apolipoproteins

The negative association of plasma HDL concentration with coronary artery disease has been well documented in epidemiological studies.1–3 Although experiments in animals have demonstrated an antiatherogenic activity of HDLs,4–8 it is not yet known whether this protective effect is related to the role of the lipoprotein in reverse cholesterol transport or to a different mechanism.9–18 Of the many components of plasma HDLs, apolipoprotein (apo) A-I appears to be critical for at least a proportion of its antiatherogenic activity.

There is now considerable support for the importance of leukocyte recruitment into the arterial intima, both for the development of atheroma and for the maintenance of the mature plaque. Indeed, the earliest observable cellular event in the genesis of atheroma is the binding of leukocytes to the endothelium.19,20 Also, several studies have now demonstrated the presence of cytokine-induced adhesion molecules for leukocytes (eg, E-selectin, intercellular adhesion molecule [ICAM]-1, and vascular cell adhesion molecule [VCAM]-1), both in animal models of atherosclerosis21,22 and in human atherosclerotic tissue.23–25 In previous studies, we have explored the possibility that the protective effect of HDLs may be related to an ability to inhibit cytokine-induced endothelial cell adhesion molecule expression. HDLs were found to inhibit the upregulation of E-selectin, VCAM-1, and ICAM-1 by interleukin (IL)-1β or tumor necrosis factor-α in cultured human umbilical vein endothelial cells at the level of both steady-state mRNA and surface protein expression.26,27 In the present study, we used a radiolabeled monoclonal antibody targeting technique in the pig, developed in our laboratory,28–30 to show that inhibition of E-selectin expression by HDLs also occurs in vivo.

**Methods**

**Reagents**

Recombinant human IL-1α (2×10^7 U/mg) was a kind gift from Glaxo Wellcome (Geneva, Switzerland). Reconstituted HDLs (recH-
DLs) were prepared from plasma-derived human apoA-I and soybean phosphatidylcholine by cholate dialysis in the ZLB Central Laboratory, resulting in the generation of discoidal particles with an average protein:phospholipid molar ratio of 1:150. The physical properties of these proteoliposomes have been described previously in detail.

Isolation and Growth of Porcine Aortic Endothelial Cells
Porcine aortic endothelial cells (PAECs) were isolated and grown according to the modified method of Jaffe et al from aortas obtained from the local abattoir and transported in Hanks’ balanced salt solution (GIBCO) containing 50 U/mL penicillin, 50 μg/mL streptomycin, 100 μg/mL gentamicin, 1 mmol/L sodium pyruvate, and 0.1 μg/mL amphoterin (all from GIBCO). After removal of loose connective tissue and ligation of branching vessels, the aortas were filled with collagenase A (0.25 mg/mL, Boehringer Mannheim) for 15 minutes at 37°C. Endothelial cells were harvested and seeded into 25-cm² flasks (Costar) precoated with 1% gelatin (Sigma) in RPMI 1640 (GIBCO) supplemented with 20% heat-inactivated FCS (GIBCO), antibiotics (as above), 15 U/mL sodium heparin, 10 mmol/L L-glutamine, and 10 μg/mL endothelial cell growth supplement (Sigma). Cells were used for experiments before the fourth passage.

Animals
The animals used in this study were healthy Large White pigs weighing 15 to 20 kg derived from lines of stock lacking the halothane-sensitivity gene. Animals were obtained from a commercial supplier and housed under standard husbandry conditions. Animals were studied according to a protocol approved under the UK Animals (Scientific Procedures) Act, 1986.

Monoclonal Antibodies
1.2B6 is a mouse IgG1 monoclonal antibody (mAb) that recognizes human E-selectin and P-selectin. This antibody also recognizes porcine E-selectin but not porcine P-selectin, as shown by reactivity with COS-7 cells transfected with porcine E-selectin or P-selectin cDNA. MOPC21 is a nonbinding mouse IgG1 myeloma protein with COS-7 cells transfected with porcine E-selectin but not porcine P-selectin, as shown by reactivity

Apo A-I Assay
ApoA-I was determined immunoturbidimetrically on a Cobas-Fara centrifugal analyzer (Roche Diagnostics). The apoA-I antibody and standards were obtained from Boehringer Mannheim. The human apoA-I antibody did not cross-react with porcine apoA-I.

Model of Cutaneous Inflammation
Animals were anesthetized for the bolus injections of recHDL and intradermal injections of IL-1α. Animals were studied according to a protocol approved under the UK Animals (Scientific Procedures) Act, 1986.

In Vivo Clearance of Reconstituted HDL
Figure 1. RecHDLs inhibit IL-1α-induced expression of E-selectin by PAECs. Concentration-dependent effect of recHDLs on IL-1α-induced expression of E-selectin by PAECs. Concentration dependence of a range of recHDLs for 3 hours. IL-1α (10 ng/mL) was then added to culture medium. Cell-surface expression of E-selectin was measured by flow cytometry 4 hours later. Values represent mean fluorescence intensities of E-selectin in response to IL (open bars) relative to unstimulated basal expression (solid bars). Values are mean±SD, n=6. Statistical analysis using 1-factor ANOVA, followed by Dunnett’s t test, P<0.01. Data are representative of 3 similar experiments.

Results
Reconstituted HDLs Inhibit Cytokine-Induced Activation of PAECs In Vitro
Before the proposed in vivo study, we needed to establish that recHDL could inhibit cytokine-induced expression of E-selectin on PAECs in vitro. When confluent monolayers of PAECs were preincubated for 3 hours with a range of concentrations of recHDL before being stimulated for 4 hours with IL-1α (10 ng/mL), the level of expression of E-selectin, measured by flow cytometry, was found to be reduced in a dose-dependent fashion (Figure 1). Maximal inhibition of 71.6±6.7% (mean±SD, P<0.01) was observed after pretreatment with recHDLs at 1 mg/mL apoA-I, compared with similar cytokine-treated cell cultures that had not been preincubated with recHDL (Figure 1).

In Vivo Clearance of Reconstituted HDL
To establish the kinetics of clearance of intravenously injected recHDL in the pig, we measured changes in the plasma concentration of human apoA-I. We found that the concentration of apoA-I had decreased by ≈30% of the initial level after 6 hours (Table).
Effect of recHDL on Basal and IL-1α-Dependent E-Selectin Expression

For our study of the effect of recHDL on expression of E-selectin in vivo, 1 pig received a bolus injection of recHDL (50 mg/kg), and a control animal received a similar volume of PBS. Fifteen minutes later, the animals were injected in multiple sites intradermally with various amounts of IL-1α in 50 μL PBS per site. It is known that unstimulated microvascular endothelium in pig skin expresses readily detectable E-selectin, and this was significantly reduced in pigs treated with recHDL, as measured by the specific uptake of intravenously injected anti-E-selectin mAb 1.2B6 after 3 hours (Figure 2). Furthermore, recHDL abrogated the upregulation of E-selectin in response to concentrations of IL-1α up to 100 ng/site.

Another experiment was conducted to exclude the possibility that the apparent inhibition of E-selectin expression by recHDL was due to interference of the binding of mAb 1.2B6 to E-selectin. Pigs were pretreated intravenously with either recHDL (50 mg/kg) or a similar volume of PBS and then were given intradermal injections of IL-1α at 1.5, 3.5, and 5.5 hours before injection of radiolabeled antibodies. During the skin spot injections, the animals recovered from sedation and were returned to holding pens. A third pig received intrave-

Discussion

Since the initial report that HDLs could inhibit cytokine-induced expression of endothelial cell adhesion molecules, further in vitro work has supported the observation by showing that HDLs containing the apoA-I_{Milano}, a naturally occurring mutant form, has similar activity and that reconstituted HDL particles containing either apoA-I or apoA-II as the sole protein can prevent cytokine-induced VCAM-1 expression. Nevertheless, there has been a need to demonstrate that HDLs can inhibit endothelial cell adhesion molecule expression in vivo. In this study we have shown, using a sensitive radiolabeled antibody targeting assay, that HDLs suppress microvascular E-selectin expression in pigs.

The only physiological way of quickly elevating the plasma HDL concentration is to give a bolus injection of discoidal apoA-I/lecithin proteoliposomes, which effectively mimics an increase in nascent HDL secretion rate by the liver. The use of phospholipid liposomes as a control would have been valid only if this had had no effect on the number and types of HDLs in plasma. However, we know that this would not have been the case. Under the influence of plasma phospholipid transfer protein, some of the infused phospholipid would have been rapidly incorporated into existing

Figure 2. Effect of recHDLs on expression of E-selectin in response to different concentrations of IL-1α. One pig received a bolus injection of recHDL (50 mg/kg) (○), and a control animal received a similar volume of PBS (□). Fifteen minutes later, animals were injected in multiple sites intradermally with various amounts of IL-1α in 50 μL of PBS per site. Animals were kept under light sedation for 3 hours before intravenous injection of radiolabeled E-selectin (mAb 1.2B6) and isotype-matched irrelevant antibody (MOPC21). Five minutes later, pigs were exsanguinated and skin disks excised and counted. Percent specific anti-E-selectin uptake was calculated as percent injected dose of E-selectin antibody localized in lesion minus percent injected dose of irrelevant control antibody. Data points are mean±SD, n=7, and are representative of 2 similar experiments (4 animals). Statistical analysis using 2-factor ANOVA, interaction P<0.05.

Figure 3. Effect of recHDL on time-dependent IL-1α-induced expression of E-selectin in porcine skin spots. Pigs were pretreated with either intravenous recHDL (50 mg/kg) (●) or a similar volume of PBS (○) and then injected intradermally with IL-1α at 2, 3.5, and 5.5 hours before injection of radiolabeled E-selectin (mAb 1.2B6) and isotope-matched irrelevant antibody (MOPC21). A third pig received intravenous recHDL (50 mg/kg) 5 minutes before injection of radiolabeled antibodies. As shown in Figure 3, no inhibition of E-selectin expression was seen in animals that received recHDL immediately before the injection of radiolabeled antibodies, indicating that recHDL did not influence the measurement of E-selectin expression with this technique. The relative distribution of both the nonspecific and the E-selectin antibodies in heart, kidney, liver, spleen, lung, and gut did not differ significantly between experiments.

Table 1. Concentration of Human Apo A-I in Pig Plasma After a Bolus Injection of recHDLs (50 mg/kg)

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HDLs. Addition of phospholipid to HDLs has been shown to destabilize the particles, leading to fusion of particles and release of apoA-I, which subsequently associates with other liposomes to produce apoA-I/phospholipid disks. Thus, the use of phospholipid liposomes as a control would not merely have increased the plasma phospholipid concentration but would also have had major effects on the number of HDLs, including the production of discoidal particles similar to those used in the test animals. The use of lipid-free apoA-I alone would also have been an unsuitable control, because this is very rapidly cleared in the plasma.

Because the original in vitro data were obtained in human endothelial cells of umbilical origin, it was important to establish that HDLs were capable of inhibiting cytokine-induced adhesion molecule expression by porcine endothelial cells before embarking on an in vivo study. We found that PAECs isolated from 50- to 60-kg animals and used at early passage were able to support a high level of expression of E-selectin on stimulation with IL-1α, as previously reported. The ability of HDLs to ablate this induction was established by preincubation of cell cultures with a physiologically relevant concentration of recHDLs.

Our results clearly indicate that recHDLs are able to reduce expression of E-selectin in vivo, both basally and in response to IL-1α. The possibility that recHDLs hindered the radiolabeled anti–E-selectin antibody from binding to its endothelial antigen was excluded by an experiment showing that recHDLs administered immediately before injection of radiolabeled antibodies had no inhibitory effect. This study therefore provides the first direct evidence that HDLs inhibit endothelial cell activation and adhesion molecule expression. Although HDL has been found to inhibit E-selectin gene transcription, the precise mechanism for this is still unknown. Because HDLs do not inhibit IκBα degradation or the nuclear translocation of nuclear factor-κB, the mechanism must involve a process independent of this ubiquitous family of transactivating factors.

In summary, we have shown for the first time that elevation of the circulating level of HDLs, in a genetically normal large mammal, can inhibit cytokine adhesion molecule expression in vivo. Our data support the anti-inflammatory function of HDLs as a potential mechanism for the recent demonstration that recA-I-Milano particles reduce macrophage infiltration in lesions in apoE-null mice, a mechanism further supported by the recent demonstration that recHDLs were also able to inhibit neointimal thickening and VCAM-1 expression in the same mouse model.

From consideration of data showing that HDLs are able to mediate differential gene expression and activate signal transduction pathways, leading to Ras activation, we hypothesize that HDLs may act through modulation of cAMP-responsive elements. We have found that HDLs modulate cAMP response element binding protein retardation complexes in endothelial cells (unpublished observation). These observations provide the basis of our future work, which may provide an explanation as to how HDLs mediate their anti-inflammatory actions. Establishing the precise mechanism of action of HDLs will be crucial if raising levels is to be considered therapeutically advantageous.

Acknowledgments

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References


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