Leukocyte-derived hepatic lipase increases HDL and decreases *en face* aortic atherosclerosis in LDLr$^{-/-}$ mice expressing CETP[S]

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Abstract In addition to hepatic expression, cholesteryl ester transfer protein (CETP) and hepatic lipase (HL) are expressed by human macrophages. The combined actions of these proteins have profound effects on HDL structure and function. It is not known how these HDL changes influence atherosclerosis. To elucidate the role of leukocyte-derived HL on atherosclerosis in a background of CETP expression, we studied low density lipoprotein receptor-deficient mice expressing human CETP (CETPtgLDLr$^{-/-}$) with a leukocyte-derived HL deficiency (HL$^{-/-}$ BM). HL$^{-/-}$ bone marrow (BM), CETPtgLDLr$^{-/-}$ mice were generated via bone marrow transplantation. Wild-type bone marrow was transplanted into CETPtgLDLr$^{-/-}$ mice to generate HL$^{+/+}$ BM, CETPtgLDLr$^{-/-}$ controls. The chimeras were fed a high-fat, high-cholesterol diet for 14 weeks to promote atherosclerosis. In female HL$^{-/-}$ BM, CETPtgLDLr$^{-/-}$ mice plasma HDL-cholesterol concentration during high-fat feeding was decreased 27% when compared with HL$^{+/+}$ BM, CETPtgLDLr$^{-/-}$ mice ($P < 0.05$), and this was associated with a 96% increase in *en face* aortic atherosclerosis ($P < 0.05$). In male CETPtgLDLr$^{-/-}$ mice, leukocyte-derived HL deficiency was associated with a 16% decrease in plasma HDL-cholesterol concentration and a 25% increase in aortic atherosclerosis. Thus, leukocyte-derived HL in CETPtgLDLr$^{-/-}$ mice has an atheroprotective role that may involve increased HDL levels.—Hime, N. J., A. S. Black, J. J. Bulgrien, and L. K. Curtiss. **Leukocyte-derived hepatic lipase increases HDL and decreases *en face* aortic atherosclerosis in LDLr$^{-/-}$ mice expressing CETP. *J. Lipid Res.* 2008.** 49: **2113–2123.**

Supplementary key words cholesteryl ester transfer protein • macrophages • high density lipoproteins • CETPtgLDLr$^{-/-}$ mice • high fat feeding

The hallmark of the atherosclerotic lesion is the lipid engorged macrophage or foam cell. These cells play a pivotal role in the progression of atherosclerotic disease via the accumulation of oxidized lipids in the intima and expression of various cytokines and chemokines (1, 2). Activated macrophages also secrete various "plasma factors," such as cholesteryl ester transfer protein (CETP), phospholipid transfer protein, hepatic lipase (HL), and lipoprotein lipase (3–9). Plasma factors act on circulatory lipoproteins and are most often investigated in this context. We propose that expression of these proteins by macrophages in the intima affects atherosclerotic disease severity (10). Supporting evidence comes from a variety of bone marrow transplantation (BMT) experiments (3, 11–13).

CETP transfers cholesteryl esters from high density lipoproteins (HDL) to triglyceride-rich lipoproteins in exchange for triglyceride (14, 15). HL hydrolyzes triglycerides and phospholipids in lipoproteins with the preferred substrate being triglyceride-enriched HDL (16). The combined in vitro actions of CETP and HL remodel HDL and generate lipid-poor apolipoprotein (apo) A-I (17), a metabolically important acceptor of cellular cholesterol via ATP-binding cassette transporter A1 (ABCA1). Thus, the combined actions of CETP and HL may have metabolic consequences on HDL that influence atherosclerotic disease. Studies in a subset of patients with elevated HDL indicate that the combined actions of CETP and HL may have antiatherosclerotic roles (18).

Published studies of atherosclerosis-prone mice where expression of either CETP or HL is only leukocyte-derived showed these proteins to be proatherogenic (3, 12). The combined effects of leukocyte-derived CETP and HL on atherosclerosis have not been studied. To examine the influence of leukocyte-derived HL on atherosclerosis in a background of CETP activity, we generated human CETP expressing, low density receptor deficient mice deficient

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in leukocyte-derived HL ($HL^{-/-}$ BM, $CETPtgLDLr^{-/-}$) via BMT. Mice were fed a high-fat, high-cholesterol diet to induce atherosclerosis. Atherosclerosis severity was measured in the whole aorta (*en face*) and the aortic sinus.

MATERIALS AND METHODS

Animals

All mice in this study were backcrossed onto a C57BL/6J background and bred in-house. LDL receptor-deficient mice ($LDLr^{-/-}$; strain B6.129S7-$Ldlr^{tm1Her}$/J) and human CETP transgenic mice (CETPtg; strain B6.CBA-Tg(CETP)5203Tall/J with natural promoter) were originally purchased from Jackson Laboratories (Bar Harbor, ME). HL-deficient mice ($HL^{-/-}$; strain B6.129P2-$Lipc^{tm1Unc}$/J) were kindly provided by Dr. Santamarina-Fojo (National Heart, Lung, and Blood Institute, NIH, Bethesda, MD). CETPtg mice deficient in the LDLr ($CETPtgLDLr^{-/-}$) and CETPtg mice deficient in HL ($CETPtgHL^{-/-}$) were generated by crossing CETPtg mice with $LDLr^{-/-}$ and $HL^{-/-}$ mice respectively. $CETPtgLDLr^{-/-}$ and $CETPtgHL^{-/-}$ mice were crossed with $LDLr^{-/-}$ and $HL^{-/-}$ mice respectively, keeping mice heterozygous for the human CETP transgene. Genotyping for $LDLr^{-/-}$, $HL^{-/-}$, and CETPtg was performed on DNA from tail clippings using protocols and primers as provided by Jackson Laboratories (http://jaxmice.jax.org/pub-cgi/protocols/protocols.sh?objtype=prot_list). The mice were weaned at 4 weeks of age and fed ad libitum a standard mouse chow (Diet No. 7019 Harlan Teklad, Madison, WI). During study periods, mice were fed a high-fat, atherogenic diet (HFD) containing 15.8% fat, 1.25% cholesterol (w/w), and no cholate (No. 94059 Harlan Teklad). All mice were housed four per cage in autoclaved, filter-top cages with autoclaved water and kept on a 12 h light/dark cycle. Animal care and use for all procedures was done in accordance with guidelines approved by the Institutional Animal Care and Use Committee of The Scripps Research Institute. This experimentation conforms with Public Health Service Policy on Humane Care and Use of Laboratory Animals.

BMT

Bone marrow transplantation (BMT) was performed as previously described (19). Briefly, bone marrow (BM)-recipient $CETPtgLDLr^{-/-}$ mice (aged 10–17 weeks) received 10 Gy γ-irradiation followed by reconstitution of BM by intravenous injection of BM containing 2×10^6 nucleated cells extracted from the femurs and tibias of 10-to 14-week-old donor mice. The donor mice were either CETPtg mice ($HL^{+/+}$ BM) or $CETPtgHL^{-/-}$ mice ($HL^{-/-}$ BM). Successful BM reconstitution was confirmed by reverse transcriptase polymerase chain reaction (RT-PCR) of blood cell lysates 5 weeks after BMT (Fig. 1). Recipient mice were fed a chow diet for 5 weeks after BMT to allow for reconstitution of BM, after which they were fed the HFD for a further 14 weeks. Periodically mice were fasted overnight, weighed, and venous blood drawn from the retro-orbital sinus into a heparinized capillary tube for determination of plasma lipids. For the assay of postheparin plasma HL activity mice were fasted and venous blood drawn 10 min after the intravenous injection of heparin (500 U/kg).

Measurement of plasma HL activity

Postheparin plasma HL activity was measured using a variation of the method described by Hocquette et al. (20) for the measurement of lipoprotein lipase. The substrate, [^{3}H]Intralipid, was prepared by adding 0.35 ml of 0.175 mCi [^{3}H]triolein (Perkin Elmer, Waltham, MA) to 0.875 ml of 20% Intralipid (Sigma, St. Louis, MO) in a glass tube. This mixture was sonicated on ice for 10 min, setting 3, 50% pulse, using a microtip attached to a sonicator (Heart Systems, Farmingdale, NY). The master mix contained [^{3}H]Intralipid and HL assay buffer (1.33M NaCl, 0.0013% heparin, 8% fatty acid free BSA, 0.2M Tris base, pH 8.5) in the ratio 1:6. Postheparin plasma (0.025 ml) was added to 15 ml polypropylene tubes. To "total cpm" and "control cpm" tubes was added 0.025 ml PBS instead of plasma. To all tubes was added 0.175 ml of master mix. Tubes were incubated in a shaking water bath for 1 h at 37°C. The incubation was stopped by adding 3.5 ml methanol:chloroform:heptane (56:50:40, v/v/v). The total cpm tube received an additional 1 ml of the solvent mixture, all other tubes received 1 ml of borate buffer (0.14 M K_2CO_3, 0.14 M H_3BO_3, pH 9.5). Tubes were vortexed vigorously and spun at 2,000 rpm for 20 min. A 500 μl aliquot of the upper, aqueous phase was added to 10 ml scintilation cocktail for counting. The percent of triglyceride hydrolyzed was determined by subtracting control cpm from sample cpm and dividing by the total cpm. Nmol of triglyceride hydrolyzed were obtained by multiplying the percent hydrolysis by the total triglyceride/tube.

Measurement of plasma CETP activity

Plasma CETP activity was determined from the transfer of fluorescent neutral lipid provided by CETP activity assay kit (BioVision, Mountain View, CA). The assay was conducted in an opaque microtiter plate with 0.005 ml of plasma and 0.195 ml of kit master mix/well. Pmol of neutral lipid transfer were determined from a standard curve of fluorescent donor molecule dissolved in isopropanol. Fluorescence intensity was measured in a luminescence spectrometer LS 55 with plate reader attachment (Perkin Elmer) at 465 nm excitation and 535 nm emission wavelengths.

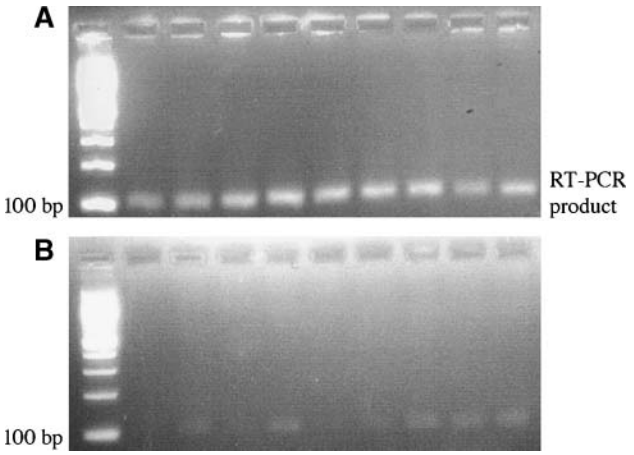

Fig. 1. Detection of the 102 base pair product of reverse transcriptase polymerase chain reaction (RT-PCR) of the hepatic lipase (HL) gene on a 3% agarose gel with ethidium bromide. Shown in A are the products of RT-PCR for blood cell lysates from nine $HL^{+/+}$ bone marrow (BM), low density lipoprotein receptor-deficient mice expressing human CETP ($HL^{+/+}$ BM, $CETPtgLDLr^{-/-}$) chimeric mice. Shown in B are the results of RT-PCR for blood cell lysates from nine $HL^{-/-}$ BM, $CETPtgLDLr^{-/-}$ chimeric mice. At left is a 100 base pair ladder with the bottom band being 100 base pairs. The 102 base pair product of RT-PCR of the HL gene is observed in A but barely detectable in B.

TABLE 1. Plasma total cholesterol, HDL-cholesterol, and apoA-I concentrations of $HL^{+/+}$ BM and $HL^{-/-}$ BM, $CETPtgLDLr^{-/-}$ chimeric mice

	Females (chow diet[a])		Males (chow diet[a])		Females (high-fat diet[b])		Males (high-fat diet[b])	
	$HL^{+/+}$ BM (n = 7)	$HL^{-/-}$ BM (n = 10)	$HL^{+/+}$ BM (n = 11)	$HL^{-/-}$ BM (n = 11)	$HL^{+/+}$ BM (n = 7)	$HL^{-/-}$ BM (n = 10)	$HL^{+/+}$ BM (n = 11)	$HL^{-/-}$ BM (n = 11)
					mg/dl			
TC	326.8 ± 17.9	319.1 ± 11.8	339.8 ± 15.5	318.6 ± 12.8	1267.0 ± 95.3	1359.4 ± 61.2	1235.7 ± 59.6	1278.6 ± 120.2
HDL-C	78.5 ± 5.1	68.4 ± 4.2	85.5 ± 3.6	79.9 ± 4.0	46.3 ± 1.6[c]	33.9 ± 3.8	57.1 ± 3.7	47.0 ± 8.6
apoA-I	17.8 ± 6.8	19.5 ± 7.6	19.0 ± 3.5	25.6 ± 6.5	20.2 ± 5.8	35.4 ± 9.3	47.7 ± 4.8	50.1 ± 13.0

$CETPtgLDLr^{-/-}$, low density lipoprotein receptor-deficient mice expressing human CETP; HDL-C, HDL-cholesterol; TC, total cholesterol. Statistical comparisons were made between $HL^{+/+}$ BM and $HL^{-/-}$ BM chimeras within the same sex and same diet.

[a] These values were obtained from a fasting blood sample drawn 5 weeks after BMT and immediately prior to the 14-week period of high-fat feeding.

[b] These values were obtained from the means of three separate fasting blood draws at 5, 10, and 14 weeks of high-fat feeding.

[c] $P < 0.05$ compared with high-fat fed female $HL^{-/-}$ BM chimeras.

Measurement of plasma total cholesterol, HDL-cholesterol and apoA-I

Plasma total cholesterol concentrations were measured by colorimetric enzymatic assay (Thermo Electron Corp., Melbourne, Australia). HDL-cholesterol concentrations were determined by first precipitating VLDL and LDL with dextran sulfate (MW 50,000, Warnick and Co.) and $MgCl_2$ (21, 22). Briefly, 0.02 ml of plasma was added to 0.06 ml of PBS and 0.008 ml of precipitating reagent (dextran sulfate, 10g/l; $MgCl_2$, 0.5 mol/l). This mixture was vortexed and incubated at room temperature for 10 min before centrifugation at 5,000 rpm for 30 min. Also, 0.04 ml of supernatant containing HDL was measured for cholesterol by colorimetric enzymatic assay. This method was validated by examining the supernatants by denaturing gel electrophoresis and Western blot for apoA-I and apoB100. ApoA-I, but not apoB100, was present in the supernatant. Plasma apoA-I levels were measured by ELISA (23).

HDL fractionation by size exclusion chromatography and apoA-I Western blotting of chromatographic fractions

Pooled plasma measuring 0.05 ml was fractioned by size exclusion chromatography on two Superdex 200 columns (Pharmacia Biotech) in series attached to an FPLC system. Fractions corresponding to HDL (eluting after VLDL and LDL) were subjected to denaturing polyacrylamide gel electrophoresis (NuPage, Invitrogen, Carlsbad, CA) and Western blotting using rabbit anti-mouse apoA-I (Biodesign International, Saco, ME). ApoA-I per chromatographic fraction was quantitated by densitometry of Western blots on a FluorChem 8900 Imager (Alpha Innotech, San Leandro, CA).

Evaluation of atherosclerosis

Atherosclerosis in the aorta from the proximal ascending aorta to the bifurcation of the iliac artery was assessed as described (19, 24). The dissected aorta was cut longitudinally under a dissecting microscope, pinned flat on black wax, and stained with Sudan IV. Pinned aortas were digitally photographed at a fixed magnification and total aortic areas and atherosclerotic lesion areas calculated using Adobe Photoshop CS2 version 9.0.2 and NIH Scion Image version 1.63 software. Results were reported as lesion area as a percentage of total *en face* aortic area. As a second assessment of atherosclerosis, lesions of the aortic root (aortic sinus) were analyzed as described (25) with modifications (24). Hearts were fixed, frozen, and sectioned on a Leica cryostat. For each aortic sinus cusp, sections were collected from the beginning of the sinus (defined as when a valve leaflet became visible) for a distance of 500 μm into the sinus. Sections (10 μm thick) were stained with oil red O and counterstained with Gill hematoxylin 1 (Fisher Scientific International). Stained sections were photographed and digitized. Lesion volume in the first 500 μm of each cusp was estimated from four sections spaced at 140 μm. Lesion volume was calculated from an integration of the measured cross-sectional areas as described (24).

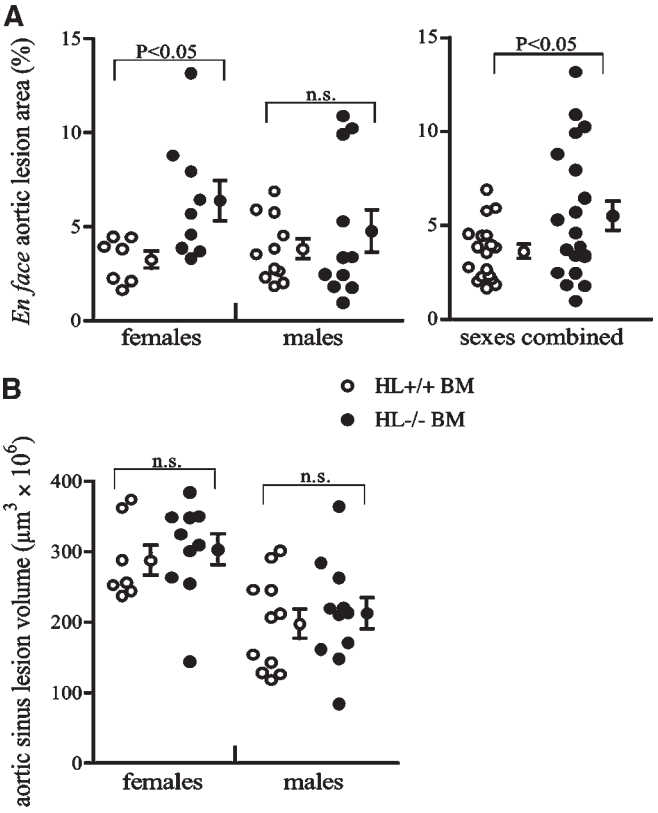

Fig. 2. Atherosclerosis severity in high-fat fed $HL^{+/+}$ BM and $HL^{-/-}$ BM, $CETPtgLDLr^{-/-}$ chimeric mice. Atherosclerosis severity was assessed in $CETPtgLDLr^{-/-}$ female and male mice transplanted with BM from either CETPtg ($HL^{+/+}$) or $CETPtgHL^{-/-}$ ($HL^{-/-}$) mice. After 14 weeks of high-fat feeding, atherosclerosis was assessed in the whole aorta (percent of *en face* aorta area that contained lesion, A) and the aortic sinus (lesion volume in the first 500 μm segment of the aortic sinus, B). Shown are measurements from individual mice as well as mean ± SEM. n.s., not significant.

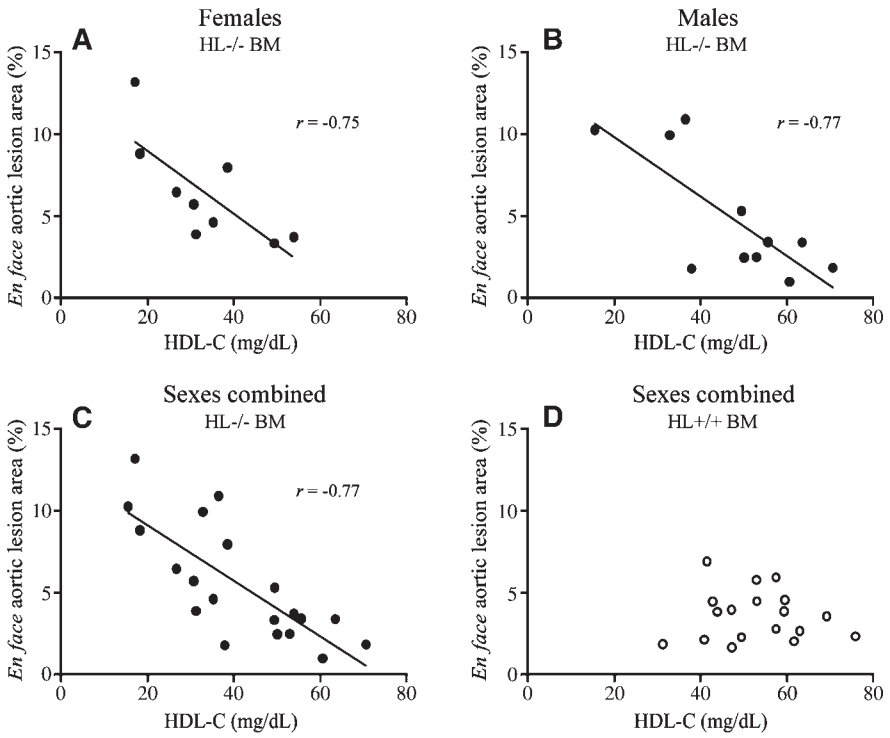

Fig. 3. Relationships between whole aortic atherosclerosis severity and plasma HDL-cholesterol (HDL-C) concentrations during high-fat feeding in $HL^{-/-}$ BM and $HL^{+/+}$ BM, CETPtgLDLr$^{-/-}$ chimeric mice. For each chimeric mouse the extent of whole aorta atherosclerosis was plotted against the mean plasma HDL-cholesterol concentration from blood draws at 5, 10, and 14 weeks of high-fat feeding. In A, B, and C are shown data for female, male, and sexes combined $HL^{-/-}$ BM, CETPtgLDLr$^{-/-}$ chimeras, respectively. Each of these data sets showed significant inverse correlations between atherosclerosis severity and plasma HDL-cholesterol concentrations. Shown in D are data for sexes combined $HL^{+/+}$ BM, CETPtgLDLr$^{-/-}$ chimeras. No significant correlation was observed in this data set. Regression lines are shown where statistically significant correlations were observed.

Data analysis and statistics

Values were expressed as mean ± SEM. Plasma cholesterol concentrations for chow-fed mice were obtained from blood collected 5 weeks after BMT and immediately before high-fat feeding. Plasma cholesterol concentrations during high-fat feeding were the mean of three values obtained 5, 10, and 14 weeks after the initiation of the HFD. A two-tailed Student's *t*-test with equal variance was used to determine significant differences in plasma cholesterol. Atherosclerosis data were analyzed with a D'Agostino and Pearson omnibus normality test using Prism

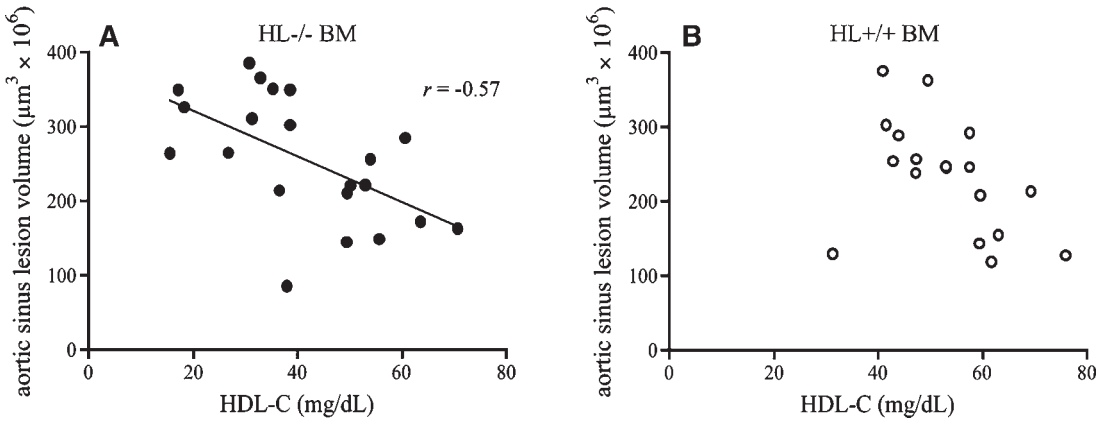

Fig. 4. Relationships between atherosclerosis severity in the aortic sinus and plasma HDL-cholesterol (HDL-C) concentrations during high-fat feeding in $HL^{-/-}$ BM and $HL^{+/+}$ BM, CETPtgLDLr$^{-/-}$ chimeric mice. For each chimeric mouse atherosclerosis severity in the aortic sinus was plotted against the mean plasma HDL-cholesterol concentration from blood draws at 5, 10, and 14 weeks of high-fat feeding. Shown in A and B are data for sexes combined for $HL^{-/-}$ BM and $HL^{+/+}$ BM, CETPtgLDLr$^{-/-}$, respectively. A regression line is shown in A, where a statistically significant correlation was observed.

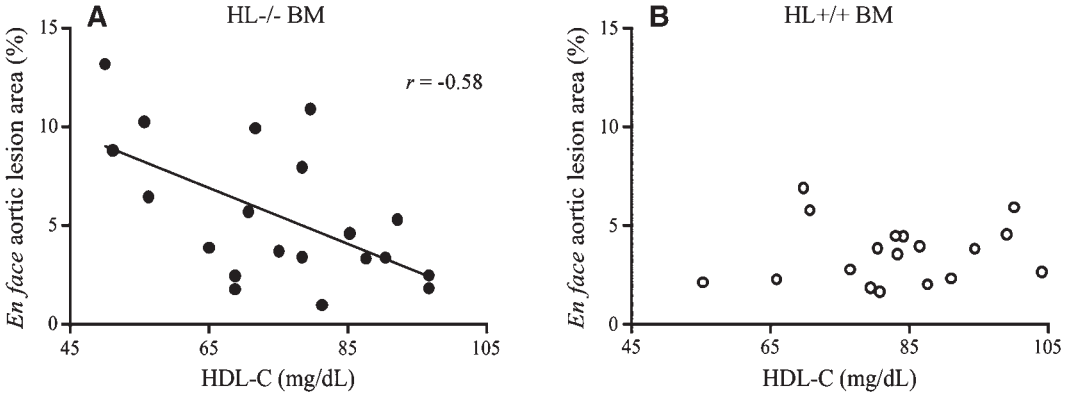

Fig. 5. Relationships between whole aorta atherosclerosis severity and chow-fed plasma HDL-cholesterol (HDL-C) concentrations in $HL^{-/-}$ BM and $HL^{+/+}$ BM, CETPtgLDLr$^{-/-}$ chimeric mice. For each chimeric mouse, atherosclerosis severity in the whole aorta was plotted against the chow-fed HDL-cholesterol concentration obtained from blood drawn immediately prior to 14 weeks of high-fat feeding. Shown in A and B are data for sexes combined for $HL^{-/-}$ BM and $HL^{+/+}$ BM, CETPtgLDLr$^{-/-}$, respectively. A regression line is shown in A, where a statistically significant correlation was observed.

4.0c software (GraphPad Software, San Diego, CA) and found to be normally distributed ($P > 0.05$). Significance was determined using a Student's *t*-test. Correlation data were also analyzed with the normality test and found to be normally distributed. The significance of correlations were determined using the Pearson's correlation test (Prism 4.0c software).

RESULTS

In this study, BMT were performed in order to examine the influence of leukocyte-derived HL in a background of systemic CETP expression. BM from either CETPtg mice ($HL^{+/+}$ BM) or CETPtgHL$^{-/-}$ mice ($HL^{-/-}$ BM) were transferred into irradiated male and female CETPtgLDLr$^{-/-}$ mice. Four weeks after BMT, HL mRNA in blood cell lysates from $HL^{-/-}$ BM chimeras as determined by RT-PCR was negligible (**Fig. 1**). The small amount of HL mRNA that was detected by the extremely sensitive RT-PCR in lysates from $HL^{-/-}$ BM chimeras is either contamination from nonblood cells or represents the small amount of rapidly dividing cells that survive irradiation. However, the difference in HL mRNA between the two chimeras is extreme. Five weeks after BMT, the chimeras began 14 weeks of high-fat feeding.

Four weeks after BMT, no statistically significant difference in postheparin plasma HL activity was observed between $HL^{-/-}$ BM (377.8 ± 22.0 nmol TG hydrolyzed/ml/h) and $HL^{+/+}$ BM (406.2 ± 28.6) chimeras. Leukocyte-derived HL deficiency also had no statistically significant effect on plasma CETP activity. In female $HL^{-/-}$ BM and $HL^{+/+}$ BM chimeras, plasma CETP activity, 5 weeks after BMT, was 1796 ± 237 and 1408 ± 166 pmol neutral lipid transfer/ml/h, respectively. In male $HL^{-/-}$ BM and $HL^{+/+}$ BM chimeras, plasma CETP activity was 2129 ± 153 and 2084 ± 100 pmol neutral lipid transfer/ml/h, respectively. Consumption of the HFD increased plasma CETP activity 4-fold as previously reported (26, 27), and there remained no influence of leukocyte-derived HL on CETP activity.

Leukocyte-derived HL deficiency had no significant effect on plasma total cholesterol, HDL-cholesterol, and

TABLE 2. Relationship between plasma HDL-cholesterol (HDL-C) concentrations and atherosclerotic disease severity in $HL^{-/-}$ BM and $HL^{+/+}$ BM, CETPtgLDLr$^{-/-}$ chimeric mice

BMT recipient mice	Parameters correlated			Result of correlation analysis for each BM chimera[a]	
				$HL^{-/-}$ BM chimera	*$HL^{+/+}$ BM chimera*
Female CETPtgLDLr$^{-/-}$	HDL-C during high-fat feeding	v	Total aortic atherosclerosis	$P < 0.05$	n.s.
Male CETPtgLDLr$^{-/-}$	HDL-C during high-fat feeding	v	Total aortic atherosclerosis	$P < 0.01$	n.s.
Sexes combined CETPtgLDLr$^{-/-}$	HDL-C during high-fat feeding	v	Total aortic atherosclerosis	$P < 0.0001$	n.s.
Female CETPtgLDLr$^{-/-}$	HDL-C during high-fat feeding	v	Aortic sinus atherosclerosis	n.s.	n.s.
Male CETPtgLDLr$^{-/-}$	HDL-C during high-fat feeding	v	Aortic sinus atherosclerosis	n.s.	n.s.
Sexes combined CETPtgLDLr$^{-/-}$	HDL-C during high-fat feeding	v	Aortic sinus atherosclerosis	$P < 0.01$	n.s.
Female CETPtgLDLr$^{-/-}$	Chow-fed HDL-C	v	Total aortic atherosclerosis	n.s.	n.s.
Male CETPtgLDLr$^{-/-}$	Chow-fed HDL-C	v	Total aortic atherosclerosis	n.s.	n.s.
Sexes combined CETPtgLDLr$^{-/-}$	Chow-fed HDL-C	v	Total aortic atherosclerosis	$P < 0.01$	n.s.

n.s., not significant. All statistically significant relationships were inverse in nature.
[a] Significance of correlations were determined by Pearson's correlation test.

apoA-I concentrations in chow-fed CETPtgLDLr$^{-/-}$ mice (**Table 1**). With high-fat feeding, plasma HDL-cholesterol concentrations were significantly reduced in leukocyte-derived HL-deficient female mice (33.9 ± 3.8 mg/dl) compared with HL$^{+/+}$ BM female chimeras (46.3 ± 1.6), $P < 0.05$. A similar trend was also observed in male mice, with HDL-cholesterol concentrations in HL$^{-/-}$ BM chimeras 47.7 ± 4.8 mg/dl compared with 57.1 ± 3.7 in HL$^{+/+}$ BM chimeras. Plasma total cholesterol and apoA-I concentrations in high-fat fed mice did not significantly differ between the BM chimeras.

The extent of atherosclerosis after 14 weeks of high-fat feeding was measured in the whole aorta (*en face*) and the aortic sinus. In female CETPtgLDLr$^{-/-}$ mice, atherosclerosis in the whole aorta was significantly greater in the HL$^{-/-}$ BM chimeras (6.4 ± 1.1% lesion area) compared with the HL$^{+/+}$ BM chimeras (3.3 ± 0.5%), $P < 0.05$ (**Fig. 2A**). The same trend of greater aortic atherosclerotic lesions in the absence of leukocyte-derived HL was observed in male CETPtgLDLr$^{-/-}$ mice, 4.8% ± 1.1% lesion area in HL$^{-/-}$ BM chimeras and 3.8 ± 0.5% in HL$^{+/+}$ BM chimeras. Overall (with BM chimeras combined) aortic atherosclerosis was not significantly different between the sexes; therefore it was considered appropriate to combine the data for the sexes. With sexes combined, the extent of aortic atherosclerosis was greater in HL$^{-/-}$ BM chimeras (5.5 ± 0.8% lesion area) compared with HL$^{+/+}$ BM chimeras (3.6 ± 0.4%), $P < 0.05$. Atherosclerosis in the aortic sinus tended toward a greater extent of disease when leukocyte-derived HL was absent without

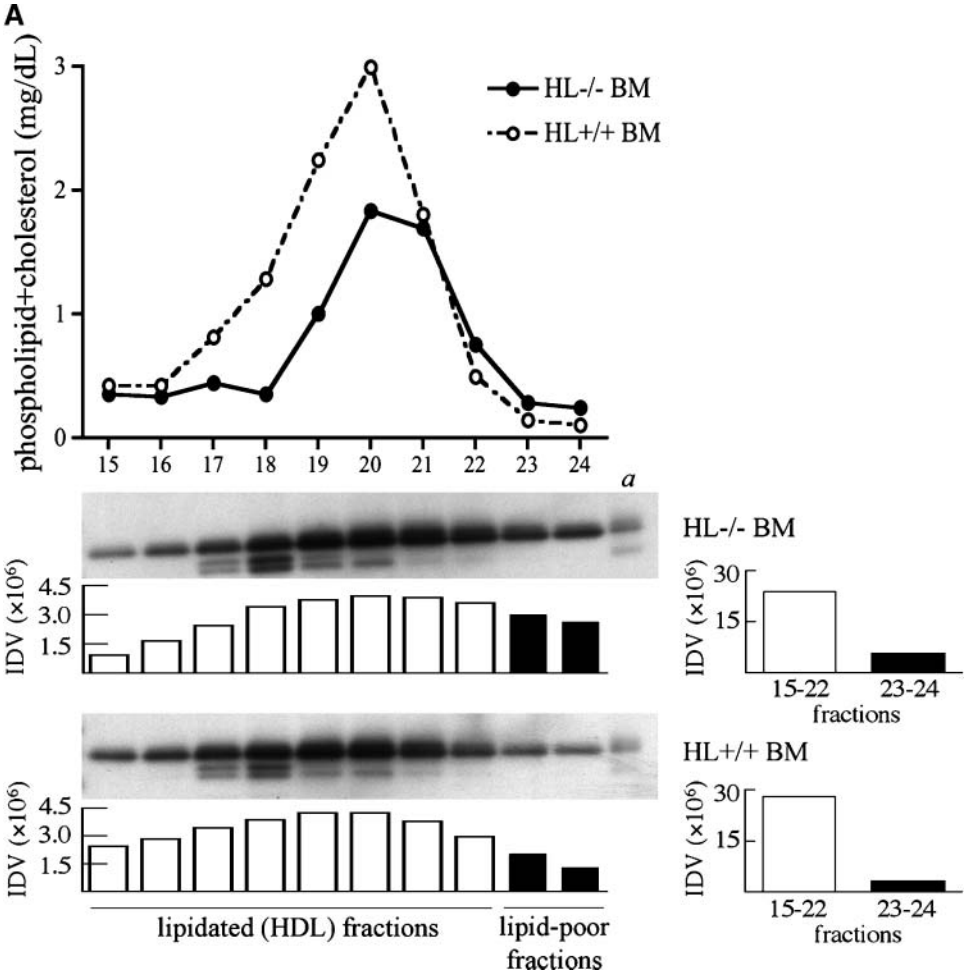

Fig. 6. Plasma lipid-poor apoA-I and lipidated (HDL) apoA-I in HL$^{-/-}$ and HL$^{+/+}$ BM, CETPtgLDLr$^{-/-}$ chimeric mice. Equal volumes of pooled plasma (7–11 mice) from HL$^{-/-}$ BM and HL$^{+/+}$ BM chimeras were subjected to size exclusion chromatography, and chromatographic fractions corresponding to HDL were assayed for phospholipid and cholesterol. Shown in A are the summed plasma phospholipid and cholesterol concentrations for these fractions from male HL$^{-/-}$ and HL$^{+/+}$ BM chimeras. By virtue of the lipid concentrations, fractions 15 to 22 were designated lipidated, and fractions 23 and 24 were designated lipid-poor. Each chromatographic fraction was Western blotted for apoA-I and the integrated density value (IDV) determined by spot densitometry as a semiquantitative measure of apoA-I level. The column graphs show the IDV for each fraction as well as the summed IDV for the lipidated and lipid-poor fractions. B shows comparable data from pooled plasma from female HL$^{-/-}$ and HL$^{+/+}$ BM chimeras. *a*, this lane of the Western blot contains purified mouse apoA-I.

reaching statistical significance (Fig. 2B). Of note, when all female mice (BM chimeras combined) were compared with all male mice, the aortic sinus lesion volume was greater in the female mice ($P = 0.0001$). It has previously been observed that atherosclerosis in the aortic sinus is greater in female than male LDLr$^{-/-}$ mice, whereas no difference is seen in the *en face* aorta (28). The reason for this is unknown; however, high-fat feeding resulted in higher plasma LDL-cholesterol concentrations in female mice than male mice (results not shown).

Because an absence of leukocyte-derived HL reduced plasma HDL-cholesterol concentrations and low plasma HDL-cholesterol is a strong predictor of atherosclerotic risk, we examined the relationship between plasma HDL-cholesterol concentrations and atherosclerotic disease severity in our chimeras. Inverse correlations between hyperlipidemic plasma HDL-cholesterol concentrations and aortic atherosclerosis severity were observed in HL$^{-/-}$ BM but not in HL$^{+/+}$ BM chimeras (**Fig. 3**). This was the case for females, males, and sexes combined, $P < 0.05$, $P < 0.01$, and $P < 0.0001$ respectively. When the relationship between hyperlipidemic plasma HDL-cholesterol concentrations and disease severity in the aortic sinus was examined, there was an inverse correlation in the sexes-combined data for HL$^{-/-}$ BM ($P < 0.01$) but not HL$^{+/+}$ BM chimeras (**Fig. 4**). Furthermore, an inverse correlation was observed between aortic atherosclerosis severity and plasma HDL-cholesterol measured in chow-fed HL$^{-/-}$ BM chimeric mice prior to high-fat feeding (**Fig. 5**, $P < 0.01$). This correlation was not seen in HL$^{+/+}$ BM chimeras. Thus, consistently inverse correlations between HDL-cholesterol concentrations and disease severity were only observed in HL$^{-/-}$ BM chimeras where there was a reduction in plasma HDL-cholesterol and not in HL$^{+/+}$ BM chimeras (**Table 2**).

Plasmas collected after 14 weeks of high-fat feeding were pooled for each BM chimera and fractionated by size exclusion chromatography. Those fractions corresponding to HDL were assayed for phospholipid and cholesterol, which represents the majority of HDL-associated lipid. Each HDL fraction was also Western blotted for apoA-I. The lanes of the blot containing the majority of apoA-I showed multiple bands that we believe are degradation products of apoA-I, as such bands were also present for purified apoA-I (**Fig. 6**, lane *a*). Previous studies have demonstrated that the combined actions of CETP and HL

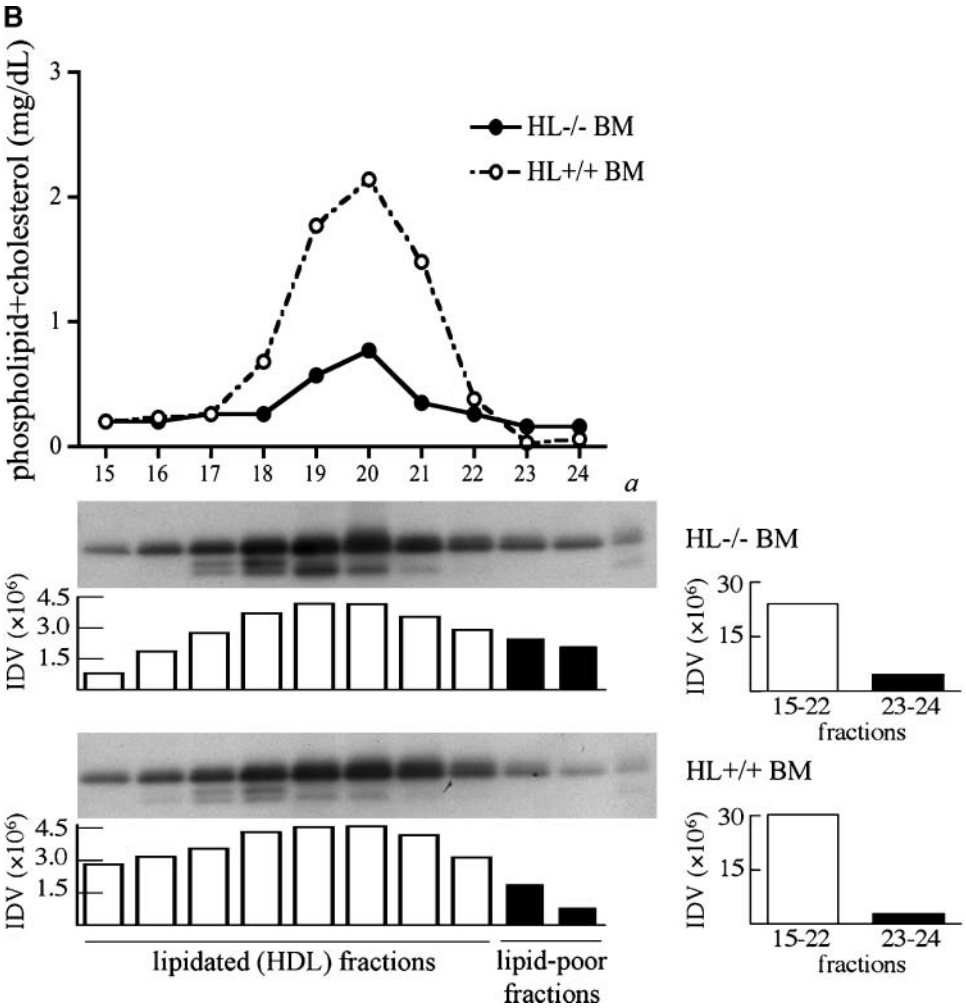

Fig. 6.—*Continued*

on HDL in vitro generate lipid-poor apoA-I (17), and we were interested to see if more lipid-poor apoA-I was present in the plasma of the $HL^{+/+}$ BM chimeras compared with $HL^{-/-}$ BM mice. Fig. 6A shows the summed cholesterol and phospholipid concentrations (for separate cholesterol and phospholipid profiles see supplementary Figure I) and spot densitometric quantitation [integrated density value (IDV)] of apoA-I Western blots for 10 chromatographic fractions (fractions 15–24) corresponding to HDL in male $HL^{-/-}$ and $HL^{+/+}$ BM chimeras. By virtue of lipid concentrations, fractions 15 to 22 were designated "lipidated" and fractions 23 and 24 were designated "lipid-poor." While fractions 15 and 16 have low lipid concentrations (like fractions 23 and 24), these fractions eluted earlier from the size exclusion column and therefore by definition contain particles that are larger in size than particles in subsequent fractions. For this reason, we felt it more appropriate to group fractions 15 and 16 with fractions 17 to 22 in the lipidated pool. In the $HL^{-/-}$ BM chimeras, the total IDV was 5.7×10^6 for the lipid-poor fractions and 23.8×10^6 for the lipidated fractions. This represents 19% of plasma apoA-I being lipid-poor. In the $HL^{+/+}$ BM chimeras, the total IDV was 3.4×10^6 for the lipid-poor fractions and 28.0×10^6 for the lipidated fractions. This represents 11% of plasma apoA-I being lipid-poor. Fig. 6B shows the lipid concentrations and spot densitometric quantitation in HDL from female $HL^{-/-}$ and $HL^{+/+}$ BM chimeras. In the $HL^{-/-}$ BM chimeras, the total IDV was 4.6×10^6 for the lipid-poor fractions and 24.1×10^6 for the lipidated fractions. This represents 16% of plasma apoA-I being lipid-poor. In the $HL^{+/+}$ BM chimeras, the total IDV was 2.7×10^6 for the lipid-poor fractions and 30.3×10^6 for the lipidated fractions. This represents 8% of plasma apoA-I being lipid-poor. Thus in male and female mice, the percentage of plasma apoA-I that was lipid-poor was 2-fold greater when leukocyte-derived HL was absent.

DISCUSSION

In this study, atherosclerosis was promoted by high-fat feeding in BM chimeric CETPtgLDLr$^{-/-}$ mice that either expressed, or were deficient in, leukocyte-derived HL. In all mice CETP was expressed in BM and non-BM cells. These chimeric models were used to determine the effect of leukocyte-derived HL deficiency on atherosclerosis in a background of systemic CETP activity. The CETPtgLDLr$^{-/-}$ mice expressed human CETP that was under the control of the natural human CETP promoter (26). While CETP expression can enhance atherosclerosis on a C57BL/6 background (29), LDLr deficiency promoted more advanced atherosclerotic lesions characteristic of human atherosclerosis.

The effect of human CETP expression on atherosclerosis in hyperlipidemic mouse models has been reported (30). The influence of leukocyte-derived HL on atherosclerosis has also been examined (3, 31). The present study differs in that the effect of leukocyte-derived HL on atherosclerosis was examined against a background of CETP expression. Numerous in vitro studies have shown that HL and CETP have direct effects on HDL form and function (32). Many epidemiologic and laboratory-based studies have demonstrated the atheroprotective properties of HDL (33–35). Furthermore, the combined effects of HL and CETP activity on HDL are associated with dyslipidemias (18, 36). These dyslipidemias may be associated with enhanced atherosclerotic risk; however it is unclear as to whether the combined actions of HL and CETP are pro-or antiatherogenic. What is known is that HL and CETP are expressed by macrophages, are present in atherosclerotic lesions (4, 37), and are therefore good candidates to influence disease development.

In this study, leukocyte-derived HL deficiency increased aortic atherosclerosis and decreased plasma HDL-cholesterol in female CETPtgLDLr$^{-/-}$ mice. This is at odds with results for previous studies. Mezdour et al. (31) found systemic HL deficiency in apoE-knockout mice decreased atherosclerosis. The systemic HL deficiency resulted in an increase in HDL-cholesterol that is not unexpected given that HL has roles in hepatic uptake and plasma clearance of HDL-cholesterol (38, 39). Thus, systemic HL deficiency increases HDL-cholesterol and decreases atherosclerosis whereas in our study leukocyte-derived HL deficiency had the opposite effect. Our study was deliberately designed to only ablate HL expression in BM cells so as not to further compromise hepatic uptake of plasma cholesterol in LDLr$^{-/-}$ mice that lack the LDLr as a means of reducing plasma cholesterol levels. In the rabbit, a model with CETP, recombinant adenovirus expression of human HL results in a reduction in HDL levels (39), and this further highlights the different effects between systemic and cell-specific HL expression. As discussed above, overexpression of HL in the rabbit is likely to have reduced HDL levels through increased hepatic uptake and plasma clearance of HDL.

The influence of macrophage-derived HL on atherosclerosis was previously examined in apoE knockout and lecithin:cholesterol acyltransferase (LCAT) transgenic mice (3). In those studies, when BM was the only source of HL, BM-derived HL promoted atherosclerotic lesion formation. When systemic HL expression was compared with BM-derived HL deficiency, disease severity was decreased in BM-derived HL-deficient mice, but only in early disease (chow-fed mice). BM-derived HL deficiency had no effect on atherosclerotic disease severity in high-fat fed mice. Furthermore, BM-derived HL deficiency had no effect on plasma HDL-cholesterol in apoE-knockout mice. Those studies differed from our study in the mouse models used (apoE knockout and LCAT transgenic vs. LDLr$^{-/-}$), and those studies were conducted in the absence of CETP. The contradictory results suggest that leukocyte-derived HL has an antiatherogenic effect of raising HDL-cholesterol, but only in the presence of CETP. In a separate study in which we examined the effect of leukocyte-derived HL deficiency in LDLr$^{-/-}$ (in the absence of CETP), we observed no change in atherosclerosis or HDL-cholesterol (data not shown). That study was con-

ducted only in male LDLr$^{-/-}$ mice; and while in male CETPtgLDLr$^{-/-}$ BM chimeras we did not observe significant differences in atherosclerosis and HDL-cholesterol, there was a trend toward decreased HDL-cholesterol and increased atherosclerosis with leukocyte-derived HL deficiency. No such trend was observed in LDLr$^{-/-}$ mice.

A discussion of the effects of CETP in mice needs to be accompanied by the caveat that mice do not normally express CETP and the protein may act differently in mice than in humans. However CETP reduces HDL-cholesterol and increases HDL-triglyceride in mice, as it does in humans, and it would be considerably difficult to conduct BMT studies in animals that naturally have CETP (hamsters, rabbits, and monkeys).

In leukocyte-derived HL-deficient female CETPtgLDLr$^{-/-}$ mice, we observed a 96% increase in aortic atherosclerosis severity and a 27% decrease in plasma HDL-cholesterol concentration. Thus, leukocyte-derived HL has significant effects on disease even in the presence of systemic HL expression. Mice (unlike humans) have high levels of circulating HL (40), making it all the more striking that leukocyte-derived HL deficiency had effects on atherosclerosis and HDL-cholesterol in the presence of systemic HL expression. This suggests that leukocyte-derived HL exerts local effects on HDL and atherosclerosis, possibly subendothelially at the site of atherosclerotic lesions.

The decrease in plasma (circulating) HDL in leukocyte-derived HL-deficient mice was somewhat surprising given that systemic expression of HL reduces circulating HDL levels (39). The reduction in HDL occurs in response to HL-mediated hydrolysis of HDL lipids and enhanced hepatic uptake of HDL. Systemic HL expression, primarily of hepatic origin, is likely to have a greater effect on plasma HDL levels than HL that is leukocyte-derived; however, this study shows that the effect of systemic HL on HDL is not so great so as to make the effects of leukocyte-derived HL insignificant. We only observed leukocyte-derived HL increasing plasma HDL when CETP was present; no effect was seen in the absence of CETP (results not shown). While previous studies examining the effect of macrophage expressed HL found no effect on circulating HDL-cholesterol levels (3), it should be stressed that those studies were conducted in the absence of CETP. It is conceivable that HL can have different effects on HDL metabolism at different anatomical sites. HL may help generate HDL in interstitial spaces where foam cells have accumulated and aid in HDL removal in the liver. These effects of increasing and decreasing plasma HDL may not necessarily be opposing as they could both help to deliver cholesterol from peripheral tissues to the liver for removal from the body in the bile.

Perhaps leukocyte-derived HL (when CETP is present) participates in HDL generation. Both the higher plasma HDL-cholesterol concentrations and lower percentage of lipid-poor apoA-I in the plasma when leukocyte-derived HL was present support this idea. In the presence of CETP, leukocyte-derived HL may facilitate the lipidation of apoA-I and thus contribute to increased plasma HDL. This appears counterintuitive as HL-mediated hydrolysis of HDL enriched in triglyceride via CETP generates lipid-free or lipid-poor apoA-I (41). However, lipid-poor apoA-I generated in the interstitial space through the activity of macrophage-derived HL would be expected to readily accept cholesterol from cholesterol-loaded macrophages via ABCA1. Thus, HL activity in the interstitial space may generate lipid-poor apoA-I locally that ultimately results in increased HDL (and an increase in the lipidation of apoA-I) in the plasma. In this way macrophage-derived

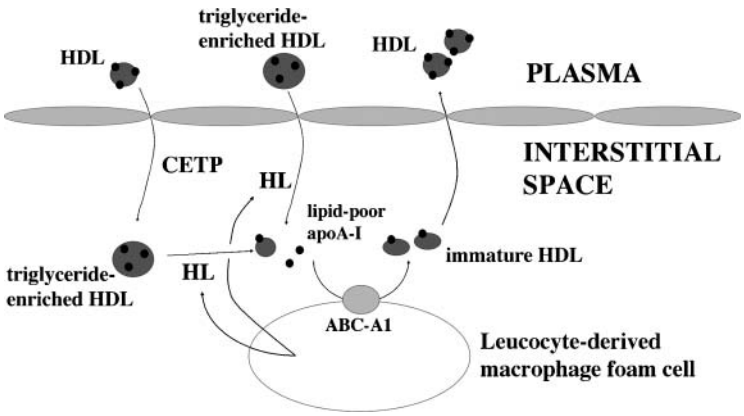

Fig. 7. Proposal for the antiatherogenic mechanism of leukocyte-derived HL in the presence of cholesteryl ester transfer protein (CETP). CETP, either in the plasma or the subendothelial interstitial space, enriches HDL in triglyceride via lipid exchange with triglyceride-rich lipoproteins. The triglyceride-enriched HDL is an ideal substrate for the lipolytic activity of HL. Leukocyte-derived HL in the interstitial space hydrolyses the phospholipid and triglyceride in the triglyceride-enriched HDL, reducing the particle size and generating lipid-poor apoA-I. The lipid-poor apoA-I is ideally situated to accept cholesterol from foam cells in atherosclerotic lesions via ATP-binding cassette transporter 1 (ABC-A1). The resulting immature HDL then enters the plasma compartment where it matures and forms new HDL particles. In this way, leukocyte-derived HL may reduce the cholesterol content and size of atherosclerotic lesions and increase the concentration of plasma HDL.

HL may have dual antiatherosclerotic effects of increasing HDL and generating lipid-poor apoA-I in the interstitial space for removal of lipid from foam cells (**Fig. 7**). This hypothesis remains to be tested ex vivo.

Triglyceride-enriched HDL are the preferred substrate for HL over HDL that are not triglyceride-enriched (16). We observed that during high-fat feeding the HDL-triglyceride:HDL-cholesterol ratio was 1:1 in CETP transgenic mice and 0.5:1 in mice without CETP (results not shown). Thus, when CETP was present, the preferred substrate for HL was available for HL-mediated generation of lipid-poor apoA-I and subsequent further HDL formation through lipidation of apoA-I via cellular lipids. This is why we believe that leukocyte-derived HL has an antiatherogenic role that is absent when CETP is not present.

The relevance of our observations to human atherosclerosis is unclear. By Western blotting, HL has been detected in protein extracts from human macrophages (37). Given that mice have basal levels of HL in the circulation (40) and humans do not, it is likely that the effects of localized (macrophage) expressed HL will be greater in humans than in mice, not less.

An inverse relationship between plasma HDL-cholesterol concentration and atherosclerotic risk is well established (33). In this study, inverse correlations between plasma HDL-cholesterol concentrations and atherosclerosis severity were only observed in leukocyte-derived HL-deficient mice. It is likely that HDL-cholesterol levels were predictive of disease severity only when HDL-cholesterol concentrations were sufficiently low to be rate limiting in relation to atheroprotective mechanisms. This occurred in leukocyte-derived HL-deficient mice, where HDL-cholesterol concentrations less than 40 mg/dl were observed, but not in mice with leukocyte-derived HL. Thus, the inverse correlations observed in leukocyte-derived HL-deficient mice was due to the greater spread of HDL-cholesterol concentrations in this group and particularly due to those mice in which HDL-cholesterol was particularly low. The inverse correlations we observed between plasma HDL-cholesterol and disease severity in leukocyte-derived HL-deficient CETPtgLDLr$^{-/-}$ mice did not establish cause and effect; however, it is likely given the extensive evidence for the atherogenicity of low HDL-cholesterol (33, 42, 43).

In summary, leukocyte-derived HL deficiency decreased plasma HDL-cholesterol and increased aortic atherosclerosis severity in hyperlipidemic female CETPtgLDLr$^{-/-}$ mice. Similar trends were observed in male CETPtgLDLr$^{-/-}$ mice. Mice were only deficient in leukocyte-derived HL; this emphasizes the importance or cell-specific, local effects of HL activity. We suggest that in the presence of CETP, leukocyte-derived HL has a role in raising plasma HDL levels (and possibly removing cholesterol from foam cells via apoA-I and ABC-A1). This would explain why we observed atheroprotection from leukocyte-derived HL, whereas other studies (in the absence of CETP) have shown leukocyte-derived HL to be atherogenic. This is a key observation given recent controversies surrounding CETP's role in disease (44), and especially considering that CETP-inhibitors have been shown to be effective antiatherosclerotic agents in rabbits (45, 46) that naturally lack HL but not in humans that have HL (47, 48).

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