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# Atheroprotective Potential of Macrophage-Derived Phospholipid Transfer Protein in Low-Density Lipoprotein Receptor–Deficient Mice Is Overcome by Apolipoprotein AI Overexpression

David T. Valenta, Nicolas Ogier, Gary Bradshaw, Audrey S. Black, David J. Bonnet, Laurent Lagrost, Linda K. Curtiss, Catherine M. Desrumaux

**Objective**—Using bone marrow transplantation, we assessed the impact of macrophage-derived phospholipid transfer protein (PLTP) on lesion development in hypercholesterolemic mice that expressed either normal levels of mouse apolipoprotein AI (apoAI) or elevated levels of only human apoAI.

**Methods and Results**—Bone marrow transplantations were performed in low-density lipoprotein receptor–deficient mice (LDLr<sup>−/−</sup>) that expressed either normal levels of mouse apoAI (*msapoAI*) or high levels of only human apoAI (*msapoAI*<sup>−/−</sup>, LDLr<sup>−/−</sup>, *huapoAITg*). Mice were lethally irradiated, reconstituted with either PLTP-expressing or PLTP-deficient bone marrow cells, and fed a high-fat diet over 16 weeks. Macrophage PLTP deficiency increased atherosclerosis in LDLr<sup>−/−</sup> mice with minimal changes in total plasma cholesterol levels. In contrast, the extent of atherosclerosis in *msapoAI*<sup>−/−</sup>, LDLr<sup>−/−</sup>, *huapoAITg* mice was not significantly different between groups that had received PLTP<sup>−/−</sup> or PLTP<sup>+/+</sup> bone marrow. In vitro studies indicated that PLTP deficiency led to a significant decrease in  $\alpha$ -tocopherol content and increased oxidative stress in bone marrow cells.

**Conclusions**—Our observations suggest an atheroprotective role of macrophage-derived PLTP in mice with normal apoAI plasma levels. The atheroprotective properties of macrophage-derived PLTP were not observable in the presence of elevated plasma concentrations of apoAI. (*Arterioscler Thromb Vasc Biol.* 2006;26:1572-1578.)

**Key Words:** phospholipid transfer protein ■ apolipoprotein AI ■ atherosclerosis ■ transgenic mice ■ bone marrow transplant

Phospholipid transfer protein (PLTP) is a multifunctional, extracellular lipid transport protein that plays a major role in phospholipid and vitamin E transfers among plasma lipoproteins as well as between lipoproteins and cell membranes.<sup>1–3</sup> In addition, PLTP participates in the formation of pre- $\beta$ -high-density lipoproteins (HDLs) that promote the efflux of excess cellular cholesterol<sup>4–6</sup> via the ATP-binding cassette transporter A1 (ABCA1) pathway.<sup>7</sup> Recent in vivo studies of PLTP transgenic and PLTP knockout mice report that PLTP plays a role in the control of plasma levels of both HDLs and apolipoprotein B (apoB)–containing lipoproteins.<sup>8–11</sup> Systemic PLTP deficiency is atheroprotective in different strains of hypercholesterolemic mice, and transgenic mice overexpressing human PLTP have an increased risk of atherosclerosis.<sup>9,12,13</sup> To further support a proatherogenic potential of plasma PLTP in vivo, a positive correlation between circulating PLTP and the risk of coronary artery disease is observed in humans.<sup>14</sup> These studies have emphasized the action of PLTP at the systemic level and suggest that its proatherogenicity is likely a result of its actions on

circulating lipoproteins. Although the impact of systemic PLTP on lipoprotein metabolism and antioxidant potential was studied, its tissue-specific actions have not been addressed.

PLTP is synthesized and secreted by most cell types in humans and mice, and although first described as a plasma protein, it was recently shown to be expressed in macrophages within the intima of human atherosclerotic arteries.<sup>15</sup> We and others reported that PLTP is synthesized and secreted by cultured macrophages, and that the gene is upregulated by liver X receptor (LXR) ligands.<sup>15,16</sup> Macrophages are essential cellular players in atherogenesis, yet it is not known whether macrophage-derived PLTP within the artery wall influences the progression of atherosclerosis. To address this question, we used bone marrow (BM) transplantation to investigate in vivo the impact of macrophage-derived PLTP on lesion progression. We found that atherosclerosis was significantly less in low-density lipoprotein receptor–deficient (LDLr<sup>−/−</sup>) mice that were reconstituted with PLTP-expressing (PLTP<sup>+/+</sup>) BM-derived cells compared with

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PLTP-deficient (PLTP<sup>-/-</sup>) BM recipients. This atheroprotective property of macrophage-derived PLTP was not observed in LDLr<sup>-/-</sup> mice that possessed large plasma concentrations of pre- $\beta$ -apoAI.

## Materials and Methods

### Animals and Facilities

All mice in this study were of a C57BL/6 background and, with the exception of PLTP<sup>-/-</sup> mice, were purchased from Jackson Laboratories (Bar Harbor, Me). Double-knockout mice deficient in mouse apoAI (*msapoAI*) and LDLr were crossed with transgenic mice with hepatic overexpression of the human apoAI gene (C57BL/6-TgN(APOA1)1Rub) to produce human apoAI-only-expressing mice (*msapoAI*<sup>-/-</sup>, LDLr<sup>-/-</sup>, *huapoAITg*). PLTP-deficient mice were a kind gift from Drs X.-C. Jiang and A.R. Tall (Columbia University, NY). The mice were fed either a chow diet (diet No. 5015; Harlan Teklad) or an atherogenic high-fat diet (HFD) containing 15.8% (w/w) fat, 1.25% (w/w) cholesterol, and no cholate (diet No. 94059; Harlan Teklad). Blood samples were collected from fasted animals by retro-orbital puncture using heparin-coated capillary tubes and transferred to EDTA-coated tubes kept on ice. All procedures were performed in accordance with institutional guidelines.

### BM Transplantation

BM transplantation (BMT) was performed as detailed previously.<sup>17</sup> Groups of 30 8- to 9-week-old male mice from each strain (LDLr<sup>-/-</sup> or *msapoAI*<sup>-/-</sup>, LDLr<sup>-/-</sup>, *huapoAITg*) were subjected to total body irradiation with a single dose of 1000 rad to eliminate stem and BM cells. Donor BM cells were extracted from tibias and femurs of PLTP<sup>-/-</sup> or control (PLTP<sup>+/+</sup>) male mice. After irradiation, 15 mice per recipient group were injected via the tail vein with  $2 \times 10^6$  PLTP<sup>-/-</sup> or PLTP<sup>+/+</sup> BM cells. The 2 recipient groups will hereafter be designated PLTP<sup>-/-</sup> BMT and PLTP<sup>+/+</sup> BMT mice, respectively. After BMT, the recipient mice were fed a regular chow diet for 4 weeks and then the HFD for an additional 16 weeks.

### Plasma Assays

Blood samples were centrifuged and the plasma collected and stored at  $-80^\circ\text{C}$  until use. Enzymatic measurement of total plasma cholesterol was performed using a colorimetric kit (Thermo). HDL cholesterol was measured after treatment with phosphotungstic acid. Human and *msapoAI* plasma levels were measured using specific ELISAs as described previously.<sup>18</sup> Plasma PLTP activity was determined with an in vitro method that measures the transfer of [<sup>14</sup>C] dipalmitoyl phosphatidylcholine (DPPC) from L- $\alpha$ -phosphatidylcholine-containing liposomes to HDLs.<sup>18</sup>

### Fast Protein Liquid Chromatography Analysis of Lipoprotein Distribution

Plasma lipoproteins were size-fractionated by fast protein liquid chromatography (FPLC) using 2 Superdex 200 columns in series. Equal volumes of plasma from 5 fasted mice were pooled and 50  $\mu\text{L}$  applied to the columns. Lipoproteins were eluted using 10 mmol/L Tris, 1 mmol/L EDTA, and 150 mmol/L NaCl buffer, pH 7.4, with 0.5-mL fractions collected. The relative cholesterol content of each fraction was measured using an enzymatic fluorescence method.<sup>17</sup> Lipoprotein distribution of *msapoAI* and human apoAI in LDLr<sup>-/-</sup> and in *msapoAI*<sup>-/-</sup>, LDLr<sup>-/-</sup>, *huapoAITg* plasma fractions, respectively, were determined by ELISA.

### Assessment of Atherosclerosis

Atherosclerosis was assessed by measuring the en face surface area of lesions across the length of aorta as well as the mean lesion area within the heart valves.<sup>17</sup> After 16 weeks of consuming the HFD, mice were euthanized, perfused, and fixed with paraformaldehyde, and the aortas and hearts extracted. Methods used to assess atherosclerosis in the aortic wall and heart valves were detailed previously.<sup>19,20</sup> Statistical differences in mean heart valve lesion area and

aortic en face lesion area between groups were calculated using the Mann-Whitney *U* test for nonparametric data.

### Cellular Cholesterol Efflux

Macrophages were harvested by peritoneal lavage with 10 mL PBS containing 10  $\mu\text{mol/L}$  butylated hydroxytoluene 4 days after intraperitoneal injection of 3 mL of 3% thioglycollate. Cells were washed and plated on 24-well plates in DMEM medium (Gibco) and supplemented with 1% Nutridoma-SP (Roche) and penicillin-streptomycin at a cell density of  $5 \times 10^5$  cells/mL. Adherent cells were recovered after 5 hours of incubation at  $37^\circ\text{C}$ .

Peritoneal macrophages were loaded with 0.5  $\mu\text{Ci/mL}$  <sup>3</sup>H-cholesterol in DMEM/2% BSA for 40 hours at  $37^\circ\text{C}$ . Cells were then incubated with DMEM/2% BSA supplemented with 100  $\mu\text{g/mL}$  of HDLs. Media were collected after 8 and 24 hours and cell debris removed. Cells were lysed in 0.5 mL of 0.1 mol/L NaOH. Radioactivity in supernatants and cell lysates was determined by liquid scintillation counting. Cell protein was measured with bicinchoninic acid reagents. Cholesterol efflux was expressed as the percentage of radioactivity released into the medium relative to total radioactivity (cells+medium) and to the amount of cell proteins in each well. Specific HDL-mediated efflux was defined as the difference between the efflux in the presence of HDLs and 2% BSA minus the efflux in the presence of only 2% BSA.

### $\alpha$ -Tocopherol Quantitation

$\alpha$ -Tocopherol was extracted and quantified by high-pressure liquid chromatography as described previously.<sup>21</sup> Tocol (Spiral) was added as an internal standard before extraction, with tocopherol contents expressed relative to cell counts determined with a hematocytometer.

### Hydroperoxide Levels in Peritoneal Macrophages and BM Cells

Cellular hydroperoxide levels were evaluated by measuring of the oxidation rate of the oxidant-sensitive dye 2,7-dichlorofluorescein diacetate (DCFH-DA) to the fluorescent product dichlorofluorescein (DCF). Peritoneal macrophages and BM cells were plated in DMEM supplemented with 1% Nutridoma-SP and penicillin-streptomycin at a cell density of  $5 \times 10^4$  cells per well. After 5 hours of incubation at  $37^\circ\text{C}$ , adherent cells were washed twice with Hanks' balanced salt solution and the medium replaced with 10  $\mu\text{g/mL}$  DCFH-DA in DMEM culture media without phenol red. The formation of DCF was monitored at excitation/emission wavelengths of 485/538 nm over 30 minutes using a VICTOR 1420 multilabel counter.

### Measurement of LDL Oxidation by Macrophages

LDL oxidation was evaluated by measurement of thiobarbituric acid-reactive substances. Peritoneal macrophages were incubated at a cell density of  $5 \times 10^5$  cells/mL for 6 hours in RPMI 1640 containing 100  $\mu\text{g/mL}$  LDL and 2.5  $\mu\text{mol/L}$  copper. Supernatants were recovered, and 0.2 mmol/L EDTA was added. One milliliter of 0.375% (wt/vol) thiobarbituric acid in 15% (v/v) trichloroacetic acid and 2% butylated hydroxytoluene were added to each sample and incubated at  $100^\circ\text{C}$  for 15 minutes. The absorbance was read at 535 nm. Results were expressed as nanomoles of malondialdehyde in LDL per microgram of cell protein.

### PLTP Activity in BM Cultures

Macrophage cultures were established from BM after a standard method<sup>22</sup> using mice that either expressed or were deficient in PLTP. Cultured cells were plated at either  $5 \times 10^5$  or  $1 \times 10^6$  cells/mL and loaded with cholesterol using serum-free DMEM media containing 1% Nutridoma-SP, acetylated LDL (Intracell), and 5  $\mu\text{mol/L}$  22-OH-cholesterol. Culture supernatants were collected after 4, 24, or 48 hours and PLTP activity determined as described above.

### Immunohistochemistry

Aortic sinus sections were immunochemically stained for the presence of macrophages using a rat monoclonal anti-mouse macrophages/

## Changes in Cholesterol and ApoAI Levels in Plasma

Strain	BM Donors		Weeks After BMT				
			4	8	12	16	20
LDLr <sup>-/-</sup>	PLTP <sup>-/-</sup>	TC	202.3±18.6	1095.4±181.4	1555.3±543.7*	1364.3±415.3	1247.6±328.4
		HDL-C	74.1±7.0	...	85.6±7.2**	...	79.8±8.9*
		Non-HDLC	128.2±11.9	...	1469.7±185.8	...	1167.8±250.3
	PLTP <sup>+/+</sup>	<i>msapoAI</i>	43.9±3.3	...	...	43.4±5.5	...
		TC	211.5±21.5	1047.3±133.9	1168.8±144.4*	1214.7±257.6	1127.2±137.8
		HDL-C	68.7±6.3	...	54.3±5.0**	...	61.4±10.3*
<i>msapoAI</i> <sup>-/-</sup> LDLr <sup>-/-</sup> <i>huapoAITg</i>	PLTP <sup>-/-</sup>	Non-HDLC	142.8±32.5	...	1114.5±160.4	...	1065.8±74.2
		<i>msapoAI</i>	42.8±3.8	...	...	39.0±2.4	...
		TC	219.6±8.2	505.9±69.9*	714.4±163.5	780.9±217.9*	565.4±88.4*
	PLTP <sup>+/+</sup>	HDL-C	122.5±32.7	...	150.3±7.0*	...	156.9±18.3
		Non-HDLC	97.1±32.9	...	564.1±34.8	...	408.5±126.1
		<i>huapoAI</i>	1172.3±361.3	...	...	1122.5±178.0	...
<i>msapoAI</i> <sup>-/-</sup> LDLr <sup>-/-</sup> <i>huapoAITg</i>	PLTP <sup>+/+</sup>	TC	216.3±80.5	567.6±64.8*	663.7±72.5	588.5±53.1*	638.3±94.3*
		HDL-C	120.6±26.7	...	125.4±9.2*	...	138.8±5.5
		Non-HDLC	95.7±41.4	...	541.3±170.2	...	499.5±78.1
		<i>huapoAI</i>	948.5±264.7	...	...	1033.7±216.9	...

Concentrations (mg/dL) of *huapoAI* and *msapoAI*, HDL (HDL-C), non-HDL (non-HDLC), and total cholesterol (TC) in plasma collected from LDLr<sup>-/-</sup> and *msapoAI*, LDLr<sup>-/-</sup>, *huapoAITg* recipient mice after transplantation of PLTP<sup>+/+</sup> BM or PLTP<sup>-/-</sup> BM.

Four weeks after BMT, the diet was changed to the HFD.

Significance levels between BM groups shown at  $P < 0.05$  (\*) and  $P < 0.001$  (\*\*).

monocytes (clone:MOMA-2) antibody (Serotec). Fluorescein isothiocyanate-conjugated anti-rat IgG was used as a secondary antibody. To visualize PLTP, cryosections were stained with a polyclonal rabbit anti-mouse PLTP antibody (Novus Biologicals). Texas Red-conjugated anti-rabbit IgG was used as a secondary antibody.

## Results

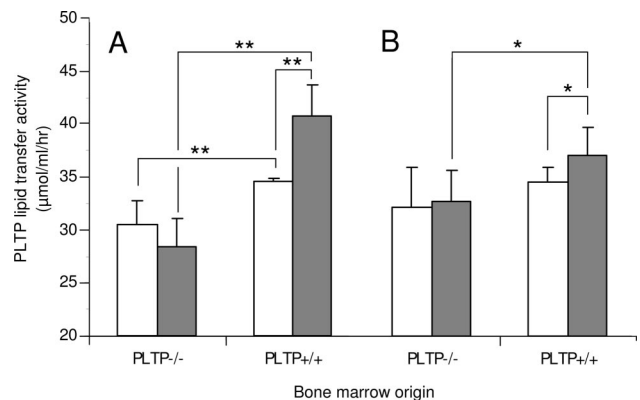
### Effect of Macrophage-Derived PLTP on Plasma Cholesterol Levels

Plasmas collected from fasted mice 4 weeks after BMT (chow diet) and at 4-week intervals while consuming the HFD were assayed for total cholesterol. When consuming the chow diet, total cholesterol levels were similar in both strains and were unaffected by BM origin (Table). After consuming the HFD for 4 weeks, cholesterol levels in LDLr<sup>-/-</sup> mice increased 5.4-fold and 4.9-fold in mice that received PLTP<sup>-/-</sup> BM and PLTP<sup>+/+</sup> BM, respectively, with no significant difference between the recipient groups. Eight weeks after consuming the HFD, total cholesterol and non-HDL cholesterol were on average higher in PLTP<sup>-/-</sup> compared with PLTP<sup>+/+</sup> BMT groups of LDLr<sup>-/-</sup> recipients. However, no significant difference appeared later on. In *msapoAI*<sup>-/-</sup>, LDLr<sup>-/-</sup>, *huapoAITg* recipient mice, a slight but significantly less pronounced increase in total cholesterol levels was recorded in PLTP<sup>-/-</sup> BMT mice (2.3-fold) compared with PLTP<sup>+/+</sup> BMT mice (2.6-fold;  $P < 0.05$ ) after 4 weeks of consuming the HFD. Twelve weeks after consuming the HFD, total cholesterol levels were significantly higher in PLTP<sup>-/-</sup> compared with PLTP<sup>+/+</sup> BMT groups, although this tendency was reversed by the end of the study (20 weeks after BMT). HDL cholesterol levels were increased ( $P < 0.05$ ) in both groups of mice that had received

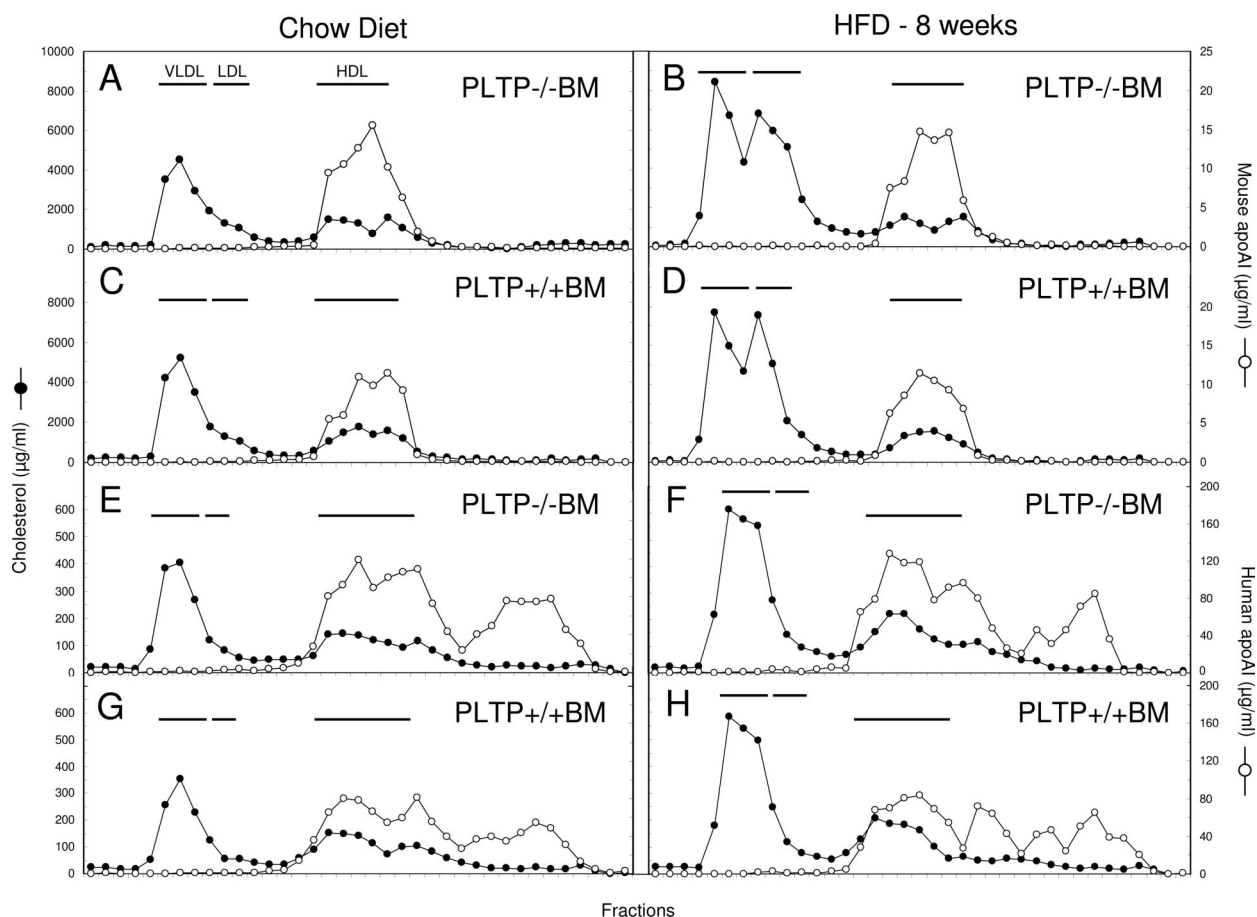
PLTP<sup>-/-</sup> BM after 12 weeks of HFD feeding compared with the PLTP<sup>+/+</sup> BM recipients. At the end of the study, this difference remained only in LDLr<sup>-/-</sup> recipient mice.

### Plasma PLTP Activity Levels

To determine the effect of macrophage-derived PLTP on plasma phospholipid transfer activity, PLTP activity was measured in plasma collected 4 and 16 weeks after BMT using an in vitro [<sup>14</sup>C]DPPC transfer assay. Four weeks after BMT, higher plasma PLTP activity levels were recorded in both recipient groups that had received PLTP<sup>+/+</sup> BM (Figure 1), confirming that stem and BM populations in the irradiated mice were successfully renewed. Sixteen weeks



**Figure 1.** Plasma PLTP activity. Plasma from LDLr<sup>-/-</sup> mice (A) and *msapoAI*<sup>-/-</sup>, LDLr<sup>-/-</sup>, *huapoAITg* mice (B) that received PLTP<sup>-/-</sup> BM or PLTP<sup>+/+</sup> BM were measured. Open bars show mice consuming a chow diet (4 weeks after BMT), and solid bars show mice 12 weeks after consuming the HFD (16 weeks after BMT). Significance levels at  $P < 0.05$  (\*) and  $P < 0.001$  (\*\*); Student *t* test.



**Figure 2.** Plasma distribution of cholesterol and apoAI. FPLC fractions of plasma were obtained from *LDLr*<sup>-/-</sup> mice (A through D) and *msapoAI*<sup>-/-</sup>, *LDLr*<sup>-/-</sup>, *huapoAITg* mice (E through H) that received PLTP<sup>-/-</sup> BM or PLTP<sup>+/+</sup> BM when consuming a chow diet and later the HFD. Total cholesterol levels were measured in 0.5-mL fractions using a fluorimetric technique; apoAI concentrations were determined by ELISA. Horizontal bars show the relative elution distribution of the major lipoprotein species.

after BMT, significantly higher plasma PLTP activity was recorded in both *LDLr*<sup>-/-</sup> and *msapoAI*<sup>-/-</sup>, *LDLr*<sup>-/-</sup>, *huapoAITg* mice that had received PLTP<sup>+/+</sup> BM compared with mice that received PLTP<sup>-/-</sup> BM. Plasma PLTP activity also increased significantly in both PLTP<sup>+/+</sup> BMT groups once they were fed an HFD, with a more notable increase in *LDLr*<sup>-/-</sup> recipient mice compared with *msapoAI*<sup>-/-</sup>, *LDLr*<sup>-/-</sup>, *huapoAITg* recipient mice.

### FPLC Fractionation of Plasma

Plasma cholesterol FPLC profiles were similar in PLTP<sup>-/-</sup> BM and PLTP<sup>+/+</sup> BM recipient mice while consuming the chow diet (Figure 2). After 8 weeks of HFD feeding, a marked increase in very low-density lipoprotein and LDL was observed in all groups, although more pronounced in *LDLr*<sup>-/-</sup> mice. In comparison, a marked increase in the HDLs was observed only in *msapoAI*<sup>-/-</sup>, *LDLr*<sup>-/-</sup>, *huapoAITg* recipient mice. The distribution of human apoAI was observed throughout the HDL particle size range, suggesting normal incorporation into HDLs in the absence of *msapoAI*. Total plasma apoAI concentrations in the plasmas of the 2 recipient strains are shown in the Table.

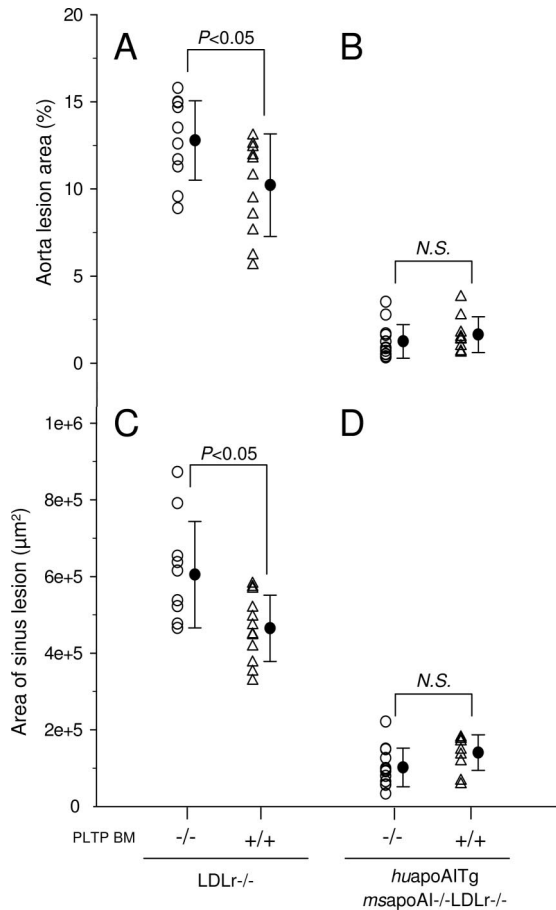
### Analysis of Atherosclerosis

Aortic and heart valve lesion areas in *LDLr*<sup>-/-</sup> mice that consumed the HFD for 16 weeks were significantly higher in

recipient mice that received PLTP<sup>-/-</sup> BM compared with PLTP<sup>+/+</sup> BM recipients (aorta  $12.8 \pm 2.28\%$  of total aorta in PLTP<sup>-/-</sup> BMT compared with  $9.99 \pm 2.67\%$  in PLTP<sup>+/+</sup> BMT mice,  $P=0.019$ ; heart valve areas  $604\,874 \pm 138\,892\ \mu\text{m}^2$  in PLTP<sup>-/-</sup> BMT compared with  $464\,565 \pm 86\,351\ \mu\text{m}^2$  in PLTP<sup>+/+</sup> BMT,  $P=0.012$ ; Figure 3). In contrast, atherosclerosis was minimal and lesions confined predominantly to the aortic arch in mice with human apoAI (*msapoAI*<sup>-/-</sup>, *LDLr*<sup>-/-</sup>, *huapoAITg*), with no significant effect of BM origin in this case (aorta  $1.25 \pm 0.96\%$  in PLTP<sup>-/-</sup> BMT compared with  $1.64 \pm 1.03\%$  in PLTP<sup>+/+</sup> BMT mice; heart valve areas  $101\,751 \pm 50,251\ \mu\text{m}^2$  in PLTP<sup>-/-</sup> BMT compared with  $140\,592 \pm 46\,447\ \mu\text{m}^2$  in PLTP<sup>+/+</sup> BMT mice).

### Cellular Cholesterol Efflux

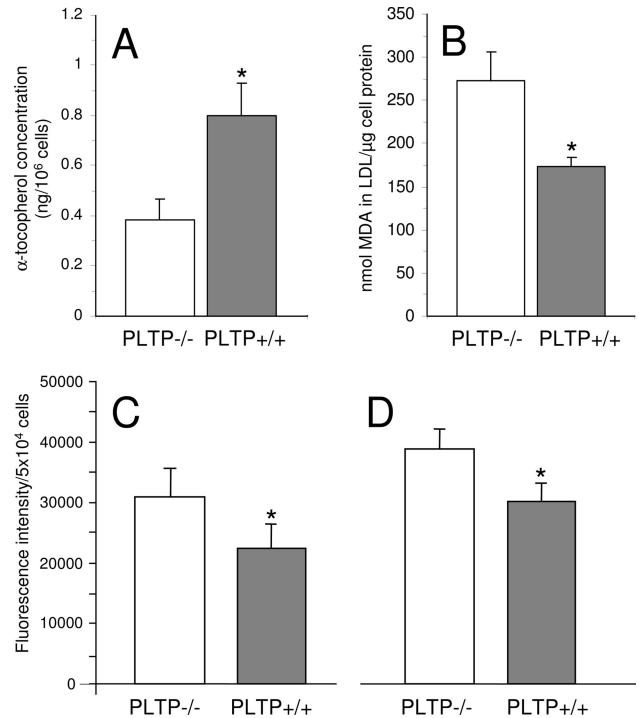
Because PLTP can promote the formation of pre- $\beta$ -HDLs or lipid-poor apoAI, we examined the impact of PLTP expression by macrophages on cholesterol efflux. After 24-hour incubation with isolated plasma HDLs, we did not observe any significant difference in cellular cholesterol efflux from PLTP<sup>+/+</sup> and PLTP<sup>-/-</sup> macrophages toward HDL particles (% cholesterol efflux/ $\mu\text{g}$  cell protein,  $0.631 \pm 0.125\%$  and  $0.676 \pm 0.092\%$  for PLTP<sup>+/+</sup> and PLTP<sup>-/-</sup> macrophages, respectively).



**Figure 3.** Atherosclerosis in BMT mice. Aortic lesion analysis in LDLr<sup>-/-</sup> (A) and *msapoAI*<sup>-/-</sup>, LDLr<sup>-/-</sup>, *huapoAITg* (B) mice transplanted with PLTP<sup>-/-</sup> BM or PLTP<sup>+/+</sup> BM and fed the HFD for 16 weeks. The analysis was performed on the luminal surface of the entire aorta after Sudan IV staining. Aortic heart valve lesion analysis in LDLr<sup>-/-</sup> (C) and *msapoAI*<sup>-/-</sup>, LDLr<sup>-/-</sup>, *huapoAITg* (D) mice transplanted with PLTP<sup>-/-</sup> BM or PLTP<sup>+/+</sup> BM and fed the HFD for 16 weeks. Statistical significance was calculated using the nonparametric Mann-Whitney *U* test.

**α-Tocopherol, Hydroperoxide, and LDL Oxidation Levels**

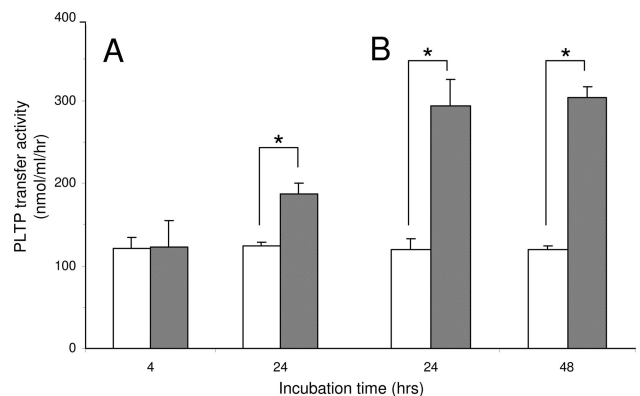
Oxidative injury to macrophages occurs in atherogenesis, and macrophage oxidative stress is a major determinant of their biological functions. Given the recently reported ability of PLTP to alter vitamin E content of various tissues,<sup>21,23–24</sup> we analyzed whether PLTP deficiency altered vitamin E levels in BM cells. We observed a significantly lower concentration of vitamin E in PLTP<sup>-/-</sup> BM cells (0.38±0.08 ng/10<sup>6</sup> cells) compared with that in PLTP-expressing cells (0.79±0.13 ng/10<sup>6</sup> cells; *P*<0.05), suggesting reduced antioxidative potential of PLTP<sup>-/-</sup> cells (Figure 4A). Isolated macrophages from PLTP<sup>-/-</sup> mice displayed a greater ability to oxidize exogenous LDL compared with macrophages from PLTP<sup>+/+</sup> mice (*P*<0.05; Figure 4B), and cellular hydroperoxides in both BM-derived and peritoneal macrophages were significantly higher in PLTP<sup>-/-</sup> compared with PLTP-expressing cells (*P*<0.05; Figure 4C and 4D).



**Figure 4.** Oxidative stress in cultured macrophages. Levels of intracellular α-tocopherol in PLTP<sup>-/-</sup> and PLTP<sup>+/+</sup> peritoneal macrophages determined by high-pressure liquid chromatography (n=8; A); oxidation of LDL assessed by measuring the formation of thiobarbituric and reactive substances in cultured peritoneal macrophages (n=8; B); and DFC measurement of intracellular hydroperoxides measured by fluorometry in peritoneal macrophages (C) and BM-derived macrophages (n=7; D). Values are mean±SEM with significance levels at *P*<0.05 (\*); Mann-Whitney *U* test.

**PLTP Activity in BM Cultures**

Cholesterol-loaded BM-derived macrophages produced and secreted active PLTP into the culture media in a cell density-dependent manner (Figure 5). Transfer activity in supernatants of PLTP<sup>-/-</sup> macrophages represented passive lipid transfer between the liposomes.



**Figure 5.** Macrophage PLTP activity. Phospholipid transfer activity by PLTP in supernatants of BM-derived macrophage cultures seeded with 5×10<sup>5</sup> cells/mL (A) and 1×10<sup>6</sup> cells/mL (B). Open bars represent PLTP<sup>-/-</sup> macrophages, and solid bars represent PLTP<sup>+/+</sup> macrophages. Significance level at *P*<0.05; Student *t* test.

### PLTP Immunostaining in Atherosclerotic Lesions

To assess whether PLTP is present in the arterial wall of recipient mice, immunostaining of aortic sinus sections from PLTP<sup>-/-</sup> and PLTP<sup>+/+</sup> BM recipient mice was performed. Double-labeling with MOMA-2 and anti-PLTP antibodies revealed that: (1) PLTP is present in atherosclerotic lesions of PLTP<sup>+/+</sup> but not PLTP<sup>-/-</sup> BM recipient mice, and (2) PLTP colocalized with macrophages in lesions (data not shown).

### Discussion

The aim of this study was to examine the impact of macrophage-derived PLTP on atherosclerotic lesion formation. We used BMT of lethally irradiated LDLr<sup>-/-</sup> mice with BM-derived cells that did or did not express the PLTP gene. LDLr<sup>-/-</sup> mice were chosen because the effect of PLTP expression on atherosclerosis is independent of the production of apoB-containing lipoproteins by the liver, which is not the case with apoE<sup>-/-</sup> or apoB/cholesterol ester transfer protein Tg mice.<sup>9</sup> Two recipient mouse strains were studied, 1 with normal apoAI levels (LDLr<sup>-/-</sup>) and a second transgenic strain that lacked *msapoAI* and overexpressed human apoAI (*msapoAI*<sup>-/-</sup>, LDLr<sup>-/-</sup>, *huapoAITg*). After challenging the mice with the HFD, we assessed the extent of atherosclerosis.

LDLr<sup>-/-</sup> mice reconstituted with macrophages that expressed PLTP developed significantly less atherosclerosis than mice reconstituted with PLTP<sup>-/-</sup> macrophages. Thus, a lack of PLTP production by BM-derived cells accelerated the progression of atherosclerosis despite the presence of PLTP in plasma. This demonstrated that unlike plasma PLTP, macrophage-derived PLTP can act as an antiatherogenic factor in lesions. Recent studies identified plasma PLTP as a novel risk factor for atherosclerosis, in which systemic PLTP deficiency in mice is associated with a decrease in atherosclerosis susceptibility, whereas overexpression of PLTP is accompanied by an increase in atherosclerotic lesions.<sup>9,12-14</sup> The antiatherogenic properties of systemic PLTP deficiency were associated with a decrease in circulating levels of apoB-containing lipoproteins as well as with a lower susceptibility of these particles to oxidation because of accumulation of vitamin E.<sup>9,21</sup> Together with these findings, our results emphasize the complexity of PLTP-directed lipoprotein metabolism and its role in atherosclerosis. Our studies provide direct in vivo evidence that the properties of PLTP with regard to atherogenesis are dependent on its site of expression.

Total plasma cholesterol levels had a limited bearing on atherosclerotic lesion formation (Table). Slight sporadic differences in total cholesterol levels were recorded between PLTP<sup>-/-</sup> BMT and PLTP<sup>+/+</sup> BMT mice in both LDLr<sup>-/-</sup> and *msapoAI*<sup>-/-</sup>, LDLr<sup>-/-</sup>, *huapoAITg* strains. However, no correlation between plasma cholesterol levels and lesion size was observed.

As expected, the levels of HDL cholesterol in plasma were considerably lower in LDLr<sup>-/-</sup> mice than in *msapoAI*<sup>-/-</sup>, LDLr<sup>-/-</sup>, *huapoAITg* mice. In addition, the influence of macrophage-derived PLTP in both strains resulted in a significant reduction in circulating HDL cholesterol when the mice were fed the HFD. ApoAI plasma concentrations, which are often tightly correlated with HDL cholesterol levels, were

unaffected by macrophage PLTP. Lipid transfer activity by PLTP in the plasma was affected by BM origin, especially in LDLr<sup>-/-</sup> mice (Figure 1), and significant increases were observed in all PLTP<sup>+/+</sup> BM recipient strains when they were fed the HFD. However, it is worthy to note that increased PLTP activity in PLTP<sup>+/+</sup> BM recipients cannot contribute to the observed decrease in atherosclerosis in these mice because circulating PLTP is recognized as a proatherogenic factor.<sup>9-14</sup>

We and others have reported that the upregulation of PLTP expression in macrophages by cholesterol loading was mediated by LXR,<sup>15,16</sup> raising the possibility that macrophage PLTP may modify lesion development. In the present study, we confirmed that cholesterol-loaded macrophages secrete significant amounts of active PLTP within the culture media after a 24-hour incubation period (Figure 5). Moreover, immunohistochemical analyses after transplantation demonstrated that: (1) PLTP is present in atherosclerotic lesions of PLTP<sup>+/+</sup> but not PLTP<sup>-/-</sup> BM recipient mice, and (2) PLTP is colocalized with macrophages in lesions. This is in accordance with previous findings in human atherosclerotic lesions.<sup>15,16</sup>

At least 2 hypotheses can explain the antiatherogenic role of macrophage-derived PLTP within the vessel wall in mice with normal but not high plasma concentrations of apoAI. First, locally produced PLTP could enhance macrophage cholesterol efflux through the generation of cholesterol-accepting pre- $\beta$ -HDL particles from spherical HDLs<sup>4-6</sup> or stabilization of ABCA1 and enhancement of ABCA1-mediated cellular cholesterol efflux.<sup>7</sup> These possibilities are discussed in detail in our recent review.<sup>25</sup> In accordance with previous findings using apoAI as cholesterol acceptor,<sup>26</sup> we found no significant difference in the ability of wild-type and PLTP<sup>-/-</sup> cells to efflux cholesterol toward HDLs. However, these studies were performed with plasma HDLs, and we published that PLTP requires triglyceride-rich HDLs to generate pre- $\beta$ -HDLs.<sup>5</sup> Further in vitro remodeling studies using triglyceride-rich HDLs are needed to better understand this process.

Because PLTP plays a major role in the transfer of  $\alpha$ -tocopherol in vivo,<sup>21,23,24</sup> a second hypothesis is that macrophage-derived PLTP may beneficially alter the distribution of  $\alpha$ -tocopherol, the main isoform of vitamin E, between lipoproteins and cells of the vascular wall. To test this, we examined whether PLTP deficiency altered vitamin E content in macrophages. We observed a significantly smaller concentration of vitamin E in PLTP<sup>-/-</sup> compared with PLTP-expressing BM cells. In addition, measurement of cellular hydroperoxides revealed increased oxidative stress in both BM cells and peritoneal macrophages from PLTP<sup>-/-</sup> compared with PLTP<sup>+/+</sup> mice (Figure 4). Finally, isolated PLTP<sup>-/-</sup> macrophages displayed a higher ability to oxidize exogenous LDL oxidation than PLTP<sup>+/+</sup> macrophages. Because oxidative injury to monocytes/macrophages is a key factor in atherogenesis,<sup>27</sup> and  $\alpha$ -tocopherol participates in the regulation of macrophage oxidative status,<sup>28</sup> abnormal cellular amounts of  $\alpha$ -tocopherol and oxidative status could also explain the proatherogenic effect of macrophage PLTP deficiency.

The present study suggests that the proatherogenic or antiatherogenic effects of PLTP are dependent on its site of action. In the periphery, the actions of PLTP are proatherogenic because it increases production of apoB-containing lipoproteins and, at the same time, depletes them of beneficial vitamin E. In contrast, locally produced PLTP within the arterial wall is antiatherogenic. We postulate that the contribution of PLTP to atherosclerosis is determined by a balance between lesion PLTP activity (antiatherogenic) and plasma PLTP activity (proatherogenic). However, these differences are confined to conditions in which plasma apoAI levels are within normal range and are not observed when high pre- $\beta$ -HDL concentrations exert an already strong antiatherogenic activity.<sup>18</sup> Further studies are needed to detail the molecular mechanisms of the antiatherogenic effect of PLTP secretion by the macrophage.

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### Disclosures

None.

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