Macrophage Phospholipid Transfer Protein Deficiency and ApoE Secretion
Impact on Mouse Plasma Cholesterol Levels and Atherosclerosis

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Objective—PLTP and apoE play important roles in lipoprotein metabolism and atherosclerosis. It is known that formation of macrophage-derived foam cells (which highly express PLTP and apoE) is the critical step in the process of atherosclerosis. We investigated the relationship between PLTP and apoE in macrophages and the atherogenic relevance in a mouse model.

Methods and Results—We transplanted PLTP-deficient mouse bone marrow into apoE-deficient mice (PLTP−/− → apoE−/−), creating a mouse model with PLTP deficiency and apoE expression exclusively in the macrophages. We found that PLTP−/− → apoE−/− mice have significantly lower PLTP activity, compared with controls (WT → apoE−/−; 20%, P<0.01). On a Western diet, PLTP−/− → apoE−/− mice have significantly lower plasma apoE than that of WT → apoE−/− mice (63%, P<0.001), and PLTP-deficient macrophages secrete significantly less apoE than WT macrophages (44%, P<0.01). Moreover, PLTP−/− → apoE−/− mice have significantly higher plasma cholesterol (98%, P<0.001) and phospholipid (107%, P<0.001) than that of WT → apoE−/− mice, thus increasing atherosclerotic lesions in the aortic arch and root (403%, P<0.001), as well as the entire aorta (298%, P<0.001).

Conclusions—Macrophage PLTP deficiency causes a significant reduction of apoE secretion from the cells, and this in turn promotes the accumulation of cholesterol in the circulation and accelerates the development of atherosclerosis. (Arterioscler Thromb Vasc Biol. 2007;27:190-196.)

Key Words: phospholipid transfer protein ▪ apoE ▪ bone marrow transplantation ▪ macrophage ▪ lipoprotein ▪ atherosclerosis

Plasma PLTP is known to be an independent risk factor for coronary artery disease, and is significantly increased in obesity, as well as in diabetes. Moreover, PLTP deficiency decreases, and PLTP overexpression increases, atherosclerosis in mouse models, so that it is considered a potential target for pharmacological or gene therapy. However, research toward this goal is hampered by the fact that the mechanism of the atherogenicity of PLTP is not completely understood. This is a multifunctional protein that is expressed in a variety of tissues, with some of its effects considered proatherogenic, and others antiatherogenic.

ApoE is a multifunctional protein that is synthesized by the liver and several peripheral tissues and cell types. ApoE serves as a ligand for receptor-mediated uptake of lipoproteins through the LDL receptor, the LDL receptor-related protein, and heparan sulfate proteoglycans. ApoE also plays a key role in intracellular lipid metabolism, influencing processes such as the assembly and secretion of lipoproteins and cholesterol efflux to HDL.

The relationship between PLTP and apoE is mostly unknown. In type 2 diabetes, PLTP activity was positively correlated with plasma apoE levels. Of the circulating PLTP mass only a minor portion is in the active form in normolipidemic subjects. It has been reported that active PLTP in plasma is associated with apoE but not with apoA-I, and apoE proteoliposomes can convert inactive PLTP into active one. There is a hypothesis that transfer of active PLTP from apoE-containing lipoproteins to apoA-I-containing ones results in the conversion of active PLTP to inactive PLTP. However, this is not confirmed by a recent report, indicating that active plasma PLTP is associated primarily with apoA-I but not apoE-containing lipoproteins.

The formation of foam cells from lipid-accumulated macrophages is a critical step in atherogenesis. Both macrophages and macrophage-derived foam cells express PLTP. It has recently been shown that PLTP is highly expressed in macrophages from atherosclerotic lesions. Macrophages synthesize and secrete apoE, which makes contribution to the apoE pool in the blood circulation and associates with plasma lipoproteins and accelerates their clearance in vivo. Macrophage-derived apoE can also act as a cholesterol acceptor to...
remove it from cholesterol-loaded cells.28,29 The effect of macrophage-derived apoE on cholesterol metabolism may be critical in protecting the artery wall from atherosclerotic lesion formation.26,27 The relationship between PLTP and apoE in macrophages is unknown. However, it has been reported that PLTP is associated with apoE in human cerebrospinal fluid, and that exogenous addition of recombinant PLTP to primary human astrocytes significantly increases apoE secretion to the conditioned medium.30 It has been also reported that PLTP secreted from HepG2 cells is associated with apoE but not apoA-I.31 These phenomena may also exist in macrophages, thus playing a role in cholesterol metabolism and the process of atherosclerosis.

To evaluate the specific relationship between PLTP and apoE in macrophages, we transplanted PLTP-deficient mouse bone marrow into apoE-deficient mice (PLTP−/− → apoE−/−), creating a model with PLTP deficiency and apoE expression exclusively in the macrophages. We investigated plasma apoE, cholesterol, and phospholipid levels, as well as atherosclerosis development in these animals.

Methods

Mice and Diets
ApoE-deficient (apoE−/−) mice (8-week-old females) of the C57BL/6 background and wild-type (WT) C57BL/6 mice were purchased from Jackson Laboratory (Bar Harbor, Me). PLTP-deficient (PLTP−/−) animals with C57BL/6 background were available in our laboratory.32 All were fed a chow diet (Research Diets, Inc). Three months after bone marrow transplantation, all mice were switched to a Western diet (0.15% cholesterol, 20% saturated fat) for 7 months. Experiments involving animals were conducted with the approval of SUNY Downstate Medical Center IACUC.

Bone Marrow Transplantation To Replace Peripheral Macrophages
Bone marrow cells were harvested from the tibias of donor mice (PLTP−/− and WT), as previously described.26,27 Twenty apoE−/− mice were lethally irradiated with 1000 rads (10 Gy). Ten of these animals were transplanted with PLTP−/− mouse bone marrow cells (5×10^6 cells), and the other 10 with WT mouse ones, via the femoral vein within 3 hours of irradiation. We monitored the process of cell replacement by polymerase chain reaction (PCR), using genomic DNA from mouse white blood cells as a template. The genotype of PLTP and apoE were determined with PCR. PLTP primer sequences: (1) TGTTGATCATCAGAACGGAAGT; (2) AAAAGCTCTGGAAGCCCGG; (3) GCACCGGATCCCTTTATCAT. ApoE primer sequences: (1) GCCTAGCCGGAGGAGACCC; (2) TGTGACT-TGGGAGCTCTGCAGC; (3) GCCGCCCCGACTGCT.

Results

PLTP Activity Assay
PLTP activity was measured with an assay kit (Cardiovascular Target, Inc) as reported previously.12

Lipid and Lipoprotein Assays
The total cholesterol and phospholipid in plasma were assayed by enzymatic methods (Wako Pure Chemical Industries, Ltd). Lipoprotein profiles were obtained by fast protein liquid chromatography (FPLC), using a Sephacryl 6B columns.33

SDS PAGE Analysis of Apolipoprotein
After 12 weeks of bone marrow transplantation, mice were fed with a Western diet for 4 weeks. Plasma was collected. The density of the plasma was adjusted to 1.21 g/mL with NaBr. Plasma lipoprotein were separated by preparative ultracentrifugation as described.37 The gel was scanned and the intensity of each band was measured by Image-Pro Plus version 4.5 software (Media Cybernetics, Inc).

Western Blot for Mouse ApoE
SDS PAGE was performed on a 4% to 20% SDS-polyacrylamide gradient gel, using 3 μL of mouse plasma or isolated lipoprotein solutions (1.006<d<1.063 g/mL and 1.063<d<1.21 g/mL, 200 μg protein), and the separated proteins were transferred to nitrocellulose membrane. Western blot analysis for mouse apoE was performed, using a polyclonal antiserum anti-apoE antibody (Santa Cruz Biotechnology, Inc). Horseradish peroxidase-conjugated rabbit polyclonal antibody to mouse IgG (Novus Biologicals) was used as a secondary antibody. The SuperSignal West detection kit (Pierce) was used for the detection step. The maximum intensity of each band was measured by Image-Pro Plus version 4.5 software (Media Cybernetics, Inc), and used for analysis.

Statistical Analysis
Each experiment was conducted at least 3 times. Data are typically expressed as mean±SD. Differences between groups were tested by Mann-Whitney U test (nonparametric test) and among multiple groups by ANOVA followed by the post-hoc test.

Results

Macrophage PLTP Deficiency, ApoE Secretion, and Atherosclerosis

The aorta was dissected and the arch photographed, as previously reported.34 Aortic root and en face assays were performed as described previously.34

Mouse Atherosclerotic Lesion Measurement
Thioglycollate-elicited peritoneal macrophages were collected by peritoneal lavage. Half million cells were suspended in Opti-Mum medium (Gibco 31985-070) and plated in a well of 24-well plate. Cells were cultured overnight and then incubated in methionine-free DMEM (Gibco 21013-024) for 20 minutes. After that the cells were incubated with 35S-methionine (40μCi/mL, Amershams Bioscences) for 6 hours. ApoE in the medium was collected by immunoprecipitation with an anti-mouse apoE antibody (Santa Cruz) and protein A/G beads (Santa Cruz), and the radioactivity was counted as described.33

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Peritoneal Macrophage Collection, Culture, and Medium ApoE Detection
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Results

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Twenty apoE−/− mice were lethally irradiated. After 3 hours, half the animals were transplanted with PLTP−/− mouse bone marrow cells (PLTP−/− apoE−/−), and the other half with WT ones (WT apoE−/−). We monitored the process of cell replacement by PCR, using genomic DNA from the mouse white blood cells as a template. In PLTP−/− apoE−/− group, by 8 weeks after transplantation, the peripheral cells had been replaced by donor cells with a PLTP deficiency and an apoE expression genotype (Figure 1). In WT apoE−/− group, the replaced peripheral cells had both a PLTP and an apoE expression genotype (Figure 1).

After 10 weeks of bone marrow transplantation, we measured plasma PLTP activity in both group of animals before and after bone marrow transplantation. We found that PLTP−/− apoE−/− mice had 20% less plasma PLTP activity than did WT apoE−/− animals, suggesting that macrophages make about a 20% contribution to PLTP activity in the circulation. Moreover, based on the results obtained from WT apoE−/− mice, we can conclude that irradiation and bone marrow transplantation did not influence plasma PLTP activity.
Plasma Lipoprotein Analysis

It is known that through replacement of apoE−/− peripheral cells with WT ones, the hyperlipidemia of these animals can be corrected, owing to the ability of macrophage-derived apoE to associate with circulating lipoproteins and promote their clearance.26,27 On chow diet, we confirmed this observation (data not shown). However, we did not find lipid changes in PLTP−/−→apoE−/− mice compared with WT→apoE−/− animals (data not shown). We next sought to determine whether a high fat high cholesterol diet could alter the lipid metabolism in those mice. After 12 weeks of bone marrow transplantation, we fed the mice with a Western diet for 4 weeks. We then determined the plasma PLTP activity and lipid levels. PLTP−/−→apoE−/− mice has 22% lower PLTP activity than that of WT→apoE−/− ones. Moreover, PLTP−/−→apoE−/− mice have significantly higher plasma cholesterol and phospholipid levels, compared with WT→apoE−/− mice (98% and 107%, P<0.001, respectively; Table). The FPLC of pooled plasma samples revealed that both non-HDL and HDL cholesterol and phospholipid were higher in PLTP−/−→apoE−/− mice than in WT→apoE−/− ones (Figure 2A and 2B). Because non-HDL lipoproteins are the major ones in the peripheral cells, the accumulated cholesterol and phospholipid are mainly located on those particles (Figure 2A and 2B). As controls, we also measured cholesterol and phospholipid distributions in WT and apoE−/− mice. Four weeks after a Western diet, WT mice accumulate cholesterol and phospholipid, most of them were on HDL fractions (Figure 2A and 2B), whereas apoE−/− mice on non-HDL fractions (Figure 2A and 2B). Assessment of apolipoprotein composition of centrifugally isolated lipoproteins by reducing SDS-PAGE revealed an increase of apoB (apoB48 + apoB100) (95%, P<0.01) and an increase of apoA-I levels (88%, P<0.01; Figure 2C through 2E). Moreover, WT→apoE−/− mice are different from WT mice, in terms of their apoB levels. ApoB48 is the major apoB in WT→apoE−/− mice (Figure 2C), whereas apoB100 is the major apoB in WT mice.37 All these results indicate that, on a Western diet, macrophage PLTP deficiency has a significant contribution to the plasma lipid and lipoprotein metabolisms.

We then sought to determine whether macrophage PLTP deficiency has an impact on apoE levels in the circulation, thus influencing the lipoprotein metabolism. We used Western blot to measure apoE in the plasma, finding that PLTP−/−→apoE−/− mice have significantly less apoE in the circulation than WT→apoE−/− (63%, P<0.01; Figure 3A and 3C). We also isolated non-HDL (1.006<d<1.063 g/mL) and HDL particles (1.063<d<1.21 g/mL) and performed the Western blot on them. We found that apoE is mainly located on non-HDL portion, and PLTP−/−→apoE−/− mouse non-HDL particles carry significant less apoE than that of WT→apoE−/− mice (70%, P<0.01; Figure 3B and 3D). There is detectable apoE on HDL portion in both PLTP−/−→apoE−/− and WT→apoE−/− mice (Figure 3B and 3E), and PLTP−/−→apoE−/− mouse HDL particles carry significant less apoE than that of WT→apoE−/− mouse (58%, P<0.01). To further evaluate the effect of PLTP deficiency on apoE in macrophage, we isolated peritoneal macrophages from both WT and PLTP−/− mice on chow or a Western diet. The macrophages were culture overnight and then pulsed with 35S-methionine for 6 hours. The radiolabeled apoE in the medium was immunoprecipitated and measured. We found that, on a Western type diet, PLTP−/− macrophage secreted significantly less apoE than WT macrophages (44%, P<0.01; Figure 3F), whereas, on chow diet, the difference did not reach statistical significance. All these results revealed that on a Western type diet PLTP deficiency in the macrophages significantly decreases apoE secretion from the cells.

Evaluation of Atherosclerosis

To evaluate the impact of macrophage PLTP deficiency on atherogenesis, we dissected mouse aortas and photographed them. We also measured proximal and whole aortic lesion areas. After 7 months on a Western diet, we found that all 10 PLTP−/−→apoE−/− mice (10/10) had very obvious lesions in the aortic arch, whereas only 2 of the 10 WT→apoE−/− animals (2/10) had observable lesions there (Figure 4A). We also found that PLTP−/−→apoE−/− mice had a 4-fold (P<0.0001) larger lesion area in the proximal aorta, and a 3-fold (P<0.0001) larger lesion area in the whole aorta, compared with WT→apoE−/− mice (Figure 4B and 4C). These results indicate that macrophage PLTP deficiency causes a significant reduction of apoE secretion from the cells, and this in turn promotes the accumulation of cholesterol in the circulation and accelerates the development of atherosclerosis.

Discussion

In this study, we found that macrophage PLTP deficiency significantly: (1) decreased PLTP activity in the circulation;
(2) increased plasma cholesterol and phospholipid levels, mainly on non-HDL lipoproteins, on a Western diet; (3) decreased plasma apoE contents which are mainly located on non-HDL lipoproteins; (4) decreased apoE secretion from peritoneal macrophages; and (5) increased atherosclerotic lesions in the aortic arch, root, and the entire aorta. It has been reported that macrophages express PLTP,23–25 but the contribution of that PLTP to the plasma PLTP has been uncertain, for other tissues including the liver, adipose tissue, lung, and small intestine, all express PLTP mRNA.35,36 Because our results show that macrophage PLTP deficiency decreases plasma PLTP activity, we know that mouse macrophage PLTP can be secreted into the circulation, making about a 20% contribution to plasma PLTP activity. However, we do not believe that 20% difference of PLTP activity in the circulation could induce such a significant lipoprotein changes (Table) and therefore atherosclerosis (Figure 4), because we know that heterozygous of PLTP deficiency (PLTP activity is decreased /H1101550%) does not change lipoprotein metabolism in mice.37

Because no evidence to show peripheral cells other than monocytes/macrophages express both PLTP and apoE, the phenomena observed in this study were coming from the PLTP-deficient and apoE-expressed monocytes/macrophages. Our results indicate that, on chow diet, there was no obvious difference between PLTP−/−→apoE−/− and WT→apoE−/− mice, in terms of lipid levels (data not shown), suggesting that the apoE in the circulation is sufficient enough for the lipid clearance. It has been reported that apoE levels, which are only 12.5% of those in normal mice, are sufficient to achieve normalization of plasma cholesterol in apoE−/− mice after WT bone marrow transplantation.26 However, when the animals were challenged with a Western diet, the PLTP−/−→apoE−/− mice cannot properly catabolize the dietary lipids owing to the defect of apoE secretion from the macrophage, thus cholesterol (mainly non-HDL cholesterol) was accumulated in the circulation (Table; Figure 2A).

The mechanism by which PLTP deficiency decreases apoE secretion from the macrophages is not yet clear. One possibility is that apoE secretion from macrophage needs PLTP assistance. It was reported that α-helix–containing apolipoproteins (apoA-I, apoA-II, apoA-IV, apoE2, apoE3, apoE4) stimulate apoE secretion, implying a positive feedback autocrine loop for apoE secretion.38 PLTP also is α-helix–containing protein39 and is involved in lipoprotein metabolism, and it is conceivable that PLTP may also be needed for proper apoE secretion from cells. Indeed, PLTP secreted from HepG2 cells is associated with apoE but not apoA-I.31 PLTP in the circulation19,20 or in human cerebrospinal fluid is associated with apoE.30 Exogenous addition of
PLTP to primary human astrocytes significantly increases apoE secretion. However, in this study, PLTP from other origin does not seem to interfere with macrophage apoE secretion, indicating that PLTP in the circulation does not but macrophage derived PLTP does directly influence apoE secretion from macrophages, although the mechanism is still unknown. Because PLTP deficiency also decreases apoB secretion from hepatocytes, there may be a PLTP-mediated mechanism for both apolipoproteins in the secreting pathway. This phenomenon deserves further investigation.

It has been reported that the increase in mouse atherosclerotic lesion area is correlated with decreased cholesterol efflux from apoE-deficient macrophages. Previous reports also indicated replacement of apoE−/− peripheral cells with WT ones, the hyperlipidemia of these animals can be corrected and atherosclerosis can be dramatically diminished, owing to apoE secretion into the circulation from macrophages. Macrophage-derived apoE per se has an antiatherogenic property. Our results revealed that macrophage PLTP deficiency blocks cellular apoE secretion and reduces apoE-mediated cholesterol clearance from the circulation, thus promoting atherosclerosis (Figure 4). In regard of apoE secretion, macrophage PLTP deficiency has a proatherogenic property.

A most recent bone marrow transplantation study indicated that PLTP−/−→LDLR−/− mice had significant more atherosclerotic lesions than WT→LDLR−/− mice. However, the proatherogenic properties of macrophage PLTP deficiency were not observable in the presence of elevated plasma concentrations of apoAI. ApoE levels were not measured in the study. It seems that a mechanism, other than prevention of apoE secretion, also make contribution to the proatherogenic properties of macrophage PLTP deficiency in mice.

It seems contradictory that macrophage-specific PLTP deficiency is proatherogenic, whereas a general PLTP deficiency is antiatherogenic. PLTP is a multifunctional protein that is expressed in a variety of tissues. Some of its effects are considered proatherogenic, and others antiatherogenic. The final atherosclerotic lesion formation is the consequence of this combination. Recent data indicate that PLTP deficiency...
in mice is associated with a decrease in atherosclerotic susceptibility, despite concomitant decreases in plasma HDL levels. Complementary metabolic studies have revealed that at least 3 distinct molecular mechanisms could account for the reduction of atherosclerosis in PLTP deficient animals. They include: (1) the reduction in liver production and plasma levels of potentially atherogenic apoB-containing lipoproteins, (2) the rise in the antioxidative potential of apoB-containing lipoproteins attributable to the accumulation of vitamin E, and (3) improvements in the antiinflammatory properties of HDL in mice, which reduce the ability of LDL to induce monocyte chemotactic activity. Published reports have also indicated that PLTP overexpression in mice increases atherosclerotic lesions, despite the increase of preβ-HDL, a known factor involved in reverse cholesterol transport.

Source of Funding
This work was supported by NIH HL-69817.

Disclosures
None.

References
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