Cell-Associated and Extracellular Phospholipid Transfer Protein in Human Coronary Atherosclerosis

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**Background**—Phospholipid transfer protein (PLTP) plays an important role in HDL particle metabolism and may modulate hepatic secretion of apolipoprotein B–containing lipoproteins. However, whether PLTP might participate directly in human atherosclerotic lesion formation is unknown.

**Methods and Results**—The cellular and extracellular distributions of PLTP were determined in normal and atherosclerotic human coronary lesions with a monoclonal antibody to human PLTP. Cell types (smooth muscle cells [SMCs] or macrophages), apolipoproteins (apoA-I, apoB, and apoE), and extracellular matrix proteoglycans (biglycan and versican) were identified on adjacent sections with monospecific antibodies. Minimal extracellular PLTP was detected in nonatherosclerotic coronary arteries, but extracellular and cellular PLTP immunostaining was widespread in atherosclerotic lesions. PLTP was detected in foam cell SMCs and in foam cell macrophages, which suggests that cellular cholesterol accumulation might increase PLTP expression in both cell types. This was confirmed by in vitro studies demonstrating that cholesterol loading of macrophages leads to 2- to 3-fold increases in PLTP steady-state mRNA levels, protein expression, and activity. PLTP also was detected in an extracellular distribution, colocalizing with apoA-I, apoB, apoE, and the vascular proteoglycan biglycan. In gel mobility shift assays, both active and inactive recombinant PLTP markedly increased HDL binding to biglycan, which suggests that PLTP may mediate lipoprotein binding to proteoglycans independent of its phospholipid transfer activity.

**Conclusions**—PLTP is present in human atherosclerotic lesions, and its distribution suggests roles for PLTP in both cellular cholesterol metabolism and lipoprotein retention on extracellular matrix. *(Circulation. 2003;108:270-274.)*

**Key Words:** lipoproteins ■ apolipoproteins ■ atherosclerosis ■ lipids ■ cells
tained from 25 hearts excised at the time of cardiac transplantation, as described previously.16 Segments were classified by morphological criteria as either atherosclerotic plaque (n=44) or nonatherosclerotic, diffuse intimal thickening (DIT, n=12).17 Morphology was determined from 6-μm sections stained with both hematoxylin and eosin or Movat’s pentachrome stains. Cells with intracellular lipid droplets identified by light microscopic examination were defined as foam cells. Segments with small amounts of immunohistochemically detected lipoprotein deposition but without macrophages were classified as DIT.

Antibodies and Antisera
Cell types were identified with monoclonal antibody HAM-56,18 which recognizes macrophages,18 and anti-α-actin, which recognizes smooth muscle cells (SMCs).19 Versican was identified with a commercially available monoclonal antibody (2B1, Seikagaku Corp),20 and biglycan was identified with rabbit polyclonal antisera (LF-51, a kind gift of Dr. Larry Fisher, National Institute of Dental Research, Bethesda, Md).18 PLTP was identified with monoclonal antibody mAb4 for immunohistochemical analysis and rabbit polyclonal antibody for Western blot analysis.21 The PLTP antibodies have been shown to be monospecific for PLTP; specifically, they do not cross-react with CETP on Western blot of human plasma.21

Single-Label Immunohistochemistry
Single-label immunohistochemistry was performed by previously described techniques16 with the following modifications. After paraffin removal and rehydration through graded alcohols, sections intended for PLTP immunostaining were pretreated with 1 mol/L urea in PBS at 95° for 20 minutes. Either 3,3'-diaminobenzidine (Vector Laboratories), which yields a brown reaction product, or Nova red (Vector), which yields a red reaction product, was used as the peroxidase substrate, and cell nuclei were counterstained with hematoxylin.

Lipoprotein Preparation
LDL (density [d]=1.019 to 1.063 g/mL), HDL1 (d=1.125 to 1.210 g/mL), and apoE-containing and apoE-free HDL3 particles were prepared as described previously.16 Acetyl-LDL was produced by the acetic anhydride method, as described previously.22

Cell Culture, Reverse Transcription–Polymerase Chain Reaction Analysis, and PLTP Activity
Human monocyte–derived macrophages were isolated from volunteer donors by the method of Böyum23 and cultured in 60-mm plastic dishes (Falcon, Becton Dickinson Labware) in RPMI-1640 medium (BioWhittaker) with 2 mmol/L-glutamine (BioWhittaker) and in dishes (Falcon, Becton Dickinson Labware) in RPMI-1640 medium the acetic anhydride method, as described previously.22

Generation of Recombinant PLTP and PLTP-Containing HDL
Recombinant human PLTP (rPLTP) was prepared as described previously.32 Six histidines were added to the carboxyl terminus to allow for rapid, 1-step affinity purification from conditioned medium with nickel-affinity chromatography (Qiagen). Also, a recombinant PLTP with low phospholipid transfer activity (r-muPLTP) was derived from a plasmid shown to contain 4 separate nucleotide substitutions that gave rise to a mutant rPLTP25 with the sequence altered at the following residues of the mature protein: L16R, L89P, Q270R, and S387P. For the gel-shift assay experiment, 100 μg of either rPLTP or r-muPLTP was incubated for 90 minutes with apoA-I HDLα at 37°C before use.

Proteoglycan Isolation and Gel Mobility Shift Assay
Biglycan16 or heparan sulfate proteoglycans (HSPGs)26 were isolated from cultures of human arterial SMCs metabolically labeled with 35S-labeled Na2SO4, as described previously.16,26 Eluted fractions were dialyzed into association buffer for the gel-shift assay. The effect of PLTP on HDLα, binding to biglycan or HSPGs was assessed with the electrophoretic gel mobility shift assay.27 Before the assay, 35S-labeled biglycan or HSPGs and HDLα with or without PLTP were dialyzed at 4°C against a buffer (Buffer A, which is adjusted to physiological pH 7.4 and contains physiological salt concentrations [20 mmol/L, MOPS buffer, 150 mmol/L NaCl, 5 mmol/L CaCl2, 2 mmol/L MgCl2]). Lipoprotein protein concentrations were determined by the Bradford assay (BioRad Laboratories), with bovine γ-globulin as the standard.

Results
Cell-Associated PLTP Is Not Detected in Control Human Coronary Arteries
PLTP protein was not detected by immunohistochemistry in either medial or intimal SMCs of normal human coronary arteries with DIT (Figure 1). This does not exclude the possibility that PLTP protein is expressed by medial or intimal SMCs at levels below the limit of detection.

PLTP Is Present in Foam Cell SMCs and Macrophages
In contrast to normal SMCs, which did not contain detectable PLTP, foam cell SMCs had abundant PLTP, both intracellular, in association with intracellular lipid droplets, and at the cell membrane (Figure 2, upper panels). It was not possible at the level of light microscopy to determine whether PLTP was associated with the inner or outer surface of the cell membrane or both. PLTP also was detected in plaque foam cell macrophages (Figure 2, lower panels) but not in non–foam cell macro-
phages. As with foam cell SMCs, macrophage PLTP was associated both with intracellular lipid droplets and at the cell membrane. This suggests that cholesterol loading may up-regulate macrophage PLTP expression in vivo.

**Macrophage PLTP mRNA, Protein, and Activity Are Increased by Cholesterol Loading In Vitro**

In Northern blotting experiments, macrophage mRNA levels were 2.0- to 2.6-fold higher in cholesterol-loaded macrophages than in control macrophages (Figure 3A). In separate RT-PCR experiments, steady-state PLTP mRNA levels were 2.2- to 2.7-fold higher in cholesterol-loaded macrophages than in control cells (Figure 3B). Western blot of conditioned media demonstrated that PLTP protein secreted into the media was 3-fold higher for cholesterol-loaded macrophages (Figure 3C). Phospholipid transfer activity was 0.16 mol·mL⁻¹·h⁻¹ in conditioned medium of control cells and was 0.35 mol·mL⁻¹·h⁻¹ in conditioned medium of cholesterol-loaded macrophages.

**Smooth muscle cell foam cells**

![A. H&E](image1)

![B. α-actin](image2)

![C. PLTP](image3)

**Macrophage foam cells**

![D. PLTP](image4)

![E. α-actin](image5)

![F. HAM-56](image6)

**Figure 1.** PLTP is not detected on SMCs of nonatherosclerotic arteries. Shown are photomicrographs of neighboring sections of nonatherosclerotic human coronary artery stained with hematoxylin and eosin (H&E; A) or immunostained with antibodies to identify SMC (α-actin; B) or PLTP (C). Arterial lumen is located at top of each photomicrograph, and arrowheads identify location of internal elastic lamina, separating intima (above) and media (below). Positive immunostaining is identified by red (B) or brown (C) reaction products. No positive immunostaining for PLTP is observed on these non–foam cell SMCs (B and C).

**Figure 2.** PLTP is expressed by foam cell SMCs and macrophages in human atherosclerotic plaques. Shown are photomicrographs showing immunostaining with antibodies to PLTP (A and D), anti-α-actin to identify SMCs (B and E), and HAM-56 to identify macrophages (C and F). Positive immunostaining is identified by brown (A and D) or red (B, C, E, and F) reaction products. Arterial lumen is located at top of each photomicrograph. Higher-power views are shown as insets in each photomicrograph. Region in upper panels (A–C) contains multiple PLTP-positive foam cells (A) that are identified as SMCs (B) but not macrophages (C). Region in lower panels (D–F) contains multiple PLTP-positive foam cells (D) that are identified as macrophages (F) but not SMCs (E). Original magnification ×400, hematoxylin counterstain.

**Figure 3.** Effect of cholesterol loading on macrophage PLTP mRNA and protein levels. A, Macrophage PLTP mRNA levels were substantially higher in cholesterol-loaded macrophages (lane 2) than in control macrophages (lane 1). B, Results of RT, followed by PCR amplification for either 21 or 23 cycles, of total RNA from macrophages cultured for 48 hours in serum-free media (lane a) or cholesterol loaded by 48-hour exposure to serum-free media containing 50 μg/mL acetyl-LDL (lane b). By this technique, steady-state mRNA levels were 2.2- to 2.7-fold higher in cholesterol-loaded macrophages. C, Western blot analysis of conditioned medium demonstrated that compared with control macrophages (lane 1), cholesterol-loaded macrophages secreted 3-fold more PLTP (lane 2). Also shown is neighboring lane with rPLTP as positive control (lane 3).

**Extracellular PLTP: Colocalization With Apolipoproteins A-I, E, and B and With Biglycan**

Extracellular PLTP was detected rarely on extracellular matrix in nonatherosclerotic DIT segments but was widespread in atherosclerotic segments, where it colocalized with apoA-I, apoE, and apoB (Figure 4). Extracellular PLTP immunostaining was not as extensive as that for extracellular apolipoproteins and was confined exclusively to extracellular lipoprotein-containing regions. This suggests that substantial amounts of lipoprotein-associated PLTP may accumulate on extracellular matrix of human atherosclerotic lesions.

Extracellular PLTP and extracellular apolipoproteins colocalized with the extracellular matrix proteoglycan biglycan.
but not with another extracellular matrix proteoglycan, versican (Figure 5). An exception to this observation was in necrotic cores, in which PLTP was present but biglycan was not. This is not surprising, because specific matrix metalloproteinases degrade extracellular matrix in necrotic cores, leaving behind lipid and its associated apolipoproteins.

**Prevalence of PLTP Distributions in Atherosclerotic and DIT Segments**

There were highly significant differences in the prevalences of each pattern of PLTP staining in atherosclerotic segments compared with DIT segments (Table). In atherosclerotic segments, extracellular PLTP was the most common pattern seen. Cell-associated PLTP also was common in atherosclerotic segments, although its prevalence was less than that of extracellular PLTP. A minority of DIT segments contained a small amount of extracellular PLTP, colocalized with biglycan in the deep intima, but cell-associated PLTP was not detected in any DIT segment.

**PLTP Mediates HDL₃ Binding to the Extracellular Matrix Proteoglycan Biglycan In Vitro**

We have shown previously that apoE-free HDL₃ particles do not bind to biglycan in vitro, which indicates that neither apoA-I nor apoA-II can mediate HDL binding to biglycan. The observation that PLTP and apolipoproteins colocalized with biglycan in human atherosclerotic lesions raised the possibility that, like apoE, PLTP might mediate HDL binding to biglycan.

This possibility was assessed in vitro with rPLTP, apoE-free HDL₃, and radiolabeled biglycan in the gel mobility shift assay. In addition, we investigated whether PLTP-mediated binding of HDL to biglycan requires phospholipid transfer activity by performing gel-shift assays with a recombinant PLTP mutant that lacks phospholipid transfer activity (r-muPLTP). As expected, apoE-free HDL₃ did not bind to biglycan (Figure 5). HDL₃ binding to biglycan was increased both by the addition of rPLTP and by r-muPLTP. The ability of rPLTP and r-muPLTP to mediate HDL₃ binding to biglycan was similar, which suggests that PLTP-mediated HDL retention on atherosclerotic extracellular matrix does not require phospholipid transfer activity.

Because most cell-surface proteoglycans are HSPGs, we also performed in vitro gel mobility shift assays with radiolabeled HSPGs and apoE-free HDL₃, with or without PLTP. The addition of PLTP led to a modest increase in binding of apoE-free HDL to HSPGs, although to a lesser extent than was seen with biglycan (data not shown).

**Discussion**

This study demonstrates the following novel findings: (1) PLTP accumulates in both cholesterol-laden macrophages and SMCs of human atherosclerotic lesions; (2) cholesterol-loading upregulates macrophage PLTP expression in vitro; (3) PLTP accumulates on extracellular matrix, colocalizing with the vascular proteoglycan biglycan; and (4) PLTP mediates HDL binding to biglycan in vitro, an effect that does not require phospholipid transfer activity.

The increased PLTP expression in foam cell macrophages both in vivo and in vitro is consistent with the hypothesis that PLTP participates in secretion of lipids from peripheral cells. In vitro, PLTP treatment of cholesterol-loaded human skin fibroblasts has shown to increase HDL binding and to enhance phospholipid and cholesterol efflux, consistent with a role for PLTP in HDL-mediated cholesterol removal from peripheral tissues. By demonstrating that PLTP content is increased in plaque cholesterol-laden macrophages and SMCs in human atherosclerosis, as well as in cholesterol-loaded macrophages in vitro, the present study suggests a similar role for PLTP in atherosclerotic plaque cellular cholesterol metabolism and raises the possibility that pharmacological inhibition of PLTP might impair cellular cholesterol efflux from atherosclerotic tissue. It is of interest that CETP has also been shown to be abundant in atherosclerotic plaque foam cells, particularly those of macrophage origin.

The present study also demonstrates that PLTP may act as a “bridging” molecule mediating binding of HDL particles to atherosclerotic plaque extracellular matrix, an effect demonstrated previously for lipoprotein lipase and apoE. Presumably, by not reentering the circulation to shuttle excess lipid from peripheral tissues back to the liver for uptake and secretion in bile, retained HDL could promote atherosclerosis. In contrast, PLTP-mediated interactions with cell-surface proteoglycans might be beneficial, for example, by increasing HDL binding to fibroblasts. In the present study, PLTP did mediate a modest increase in HDL₃ binding to secreted HSPGs in vitro.
The present study also suggests that both active and inactive PLTP mediates lipoprotein retention on vascular proteoglycans, specifically biglycan. This is important because a substantial proportion of circulating human PLTP lacks significant phospholipid transfer activity.\textsuperscript{21,32} Although the inactive PLTP protein used in the present study is not a naturally occurring mutant, PLTP and the inactive mutant PLTP were equally effective at mediating HDL retention. On the basis of studies demonstrating that atherosclerosis is decreased in PLTP-deficient mice,\textsuperscript{15} it has been proposed that pharmacological inhibition of PLTP activity may inhibit atherosclerosis. However, PLTP-deficient mice not only lack PLTP activity, they also lack the protein itself. This raises the possibility that even in the presence of pharmacological inhibitors of its activity, PLTP could still promote lipoprotein retention on extracellular matrix.

In summary, the present study suggests several potential roles for PLTP in human atherosclerosis. Roles for PLTP in cellular cholesterol removal are suggested by the observations that PLTP content is increased in lipid-laden macrophages and SMCs in human atherosclerotic plaques and that cholesterol loading increases PLTP expression in cultured macrophages. In addition, the results suggest a structural role for PLTP in mediating lipoprotein retention on extracellular matrix proteoglycans. Further studies aimed at dissecting the cellular and extracellular roles of PLTP in atherosclerotic plaque cholesterol metabolism are warranted.

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**References**

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