Phospholipid Transfer Protein Is Present in Human Tear Fluid†

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Received January 26, 2005; Revised Manuscript Received April 13, 2005

ABSTRACT: The human tear fluid film consists of a superficial lipid layer, an aqueous middle layer, and a hydrated mucin layer located next to the corneal epithelium. The superficial lipid layer protects the eye from drying and is composed of polar and neutral lipids provided by the meibomian glands. Excess accumulation of lipids in the tear film may lead to drying of the corneal epithelium. In the circulation, phospholipid transfer protein (PLTP) and cholesteryl ester transfer protein (CETP) mediate lipid transfers. To gain insight into the formation of tear film, we investigated whether PLTP and CETP are present in human tear fluid. Tear fluid samples were collected with microcapillaries. The presence of PLTP and CETP was studied in tear fluid by Western blotting, and the PLTP concentration was determined by ELISA. The activities of the enzymes were determined by specific lipid transfer assays. Size-exclusion and heparin-affinity chromatography assessed the molecular form of PLTP. PLTP is present in tear fluid, whereas CETP is not. Quantitative assessment of PLTP by ELISA indicated that the PLTP concentration in tear fluid, 10.9 ± 2.4 μg/mL, is about 2-fold higher than that in human plasma. PLTP-facilitated phospholipid transfer activity in tears, 15.1 ± 1.8 μmol mL^{-1} h^{-1}, was also significantly higher than that measured in plasma. Inactivation of PLTP by heat treatment (+58 °C, 60 min) or immunoinhibition abolished the phospholipid transfer activity in tear fluid. Size-exclusion chromatography of tear fluid indicated that PLTP eluted in a position corresponding to a size of 160−170 kDa. Tear fluid PLTP was quantitatively bound to Heparin−Sepharose and could be eluted as a single peak by 0.5 M NaCl. These data indicate that human tear fluid contains catalytically active PLTP protein, which resembles the active form of PLTP present in plasma. The results suggest that PLTP may play a role in the formation of the tear film by supporting phospholipid transfer.

The tear film is a layered gel-like structure consisting of a superficial lipid layer, an aqueous layer, and a deep hydrated mucin layer (*1*). The total thickness of the tear film ranges from 3 to 45 μm depending upon the investigative method (reviewed in ref *2*). The chemical composition of the tear film is not known, and no comprehensive proteomic analysis of tear fluid has been published. On the basis of the analysis of meibomian gland secretions, the superficial lipid layer has been suggested to be composed of wax esters, sterol esters, and polar lipids (*3*, *4*).

Keratoconjunctivitis sicca (KC)[1] or dry eye syndrome is a common ocular disorder. A characteristic feature of KC is corneal epithelial damage because of drying of the ocular surface. This may lead to permanent corneal scarring or even perforation of the eye. KC is considered to arise from the interplay between an inadequate tear production, increased tear evaporation, and altered composition of the tear film. The underlying molecular events remain incompletely understood (*5*).

Phospholipid transfer protein (PLTP) and cholesteryl ester transfer protein (CETP) play an important role in plasma lipoprotein metabolism. PLTP is a glycoprotein that was originally identified based on its capability to transfer phospholipids but not neutral lipids between phospholipid vesicles and HDL (*6*). PLTP is also capable of converting HDL_3 into large HDL particles via a fusion process with a concomitant generation of small preβ-HDL particles (*7*, *8*). CETP is a 74-kDa hydrophobic glycoprotein circulating bound to HDL (*9*). The major function of CETP in plasma is to facilitate the transfer of cholesteryl esters from HDL to apolipoprotein B-containing lipoproteins and the reciprocal transfer of triglycerides (*10*). PLTP shows sequence homol-

† The work was supported by the Mary and Georg C. Ehrnrooth Foundation, the Ella and Georg Ehrnrooth Foundation, the Finnish Eye Foundation, the Finnish Foundation for Cardiovascular Research (to M.J.), Sigrid Juselius Foundation (to M.J.), International HDL Research Award (to C.E. and M.J.) and the Finska Läkaresällskapet (to O.E.).

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[1] Abbreviations: CETP, cholesteryl ester transfer protein; HA-PLTP, high-activity phsopholipid transfer protein; KC, keratoconjunctivitis sicca; LA-PLTP, low-activity phospholipid transfer protein; PBS, phosphate-buffered saline; PLTP, phospholipid transfer protein.

10.1021/bi050151k CCC: $30.25
Published on Web 05/13/2005

ogy to three other members of the lipopolysaccharide (LPS)-binding/lipid transfer protein family, i.e., CETP, lipopolysaccharide binding protein (LBP), and bactericidal permeability increasing protein (BPI) (*11*, *12*). The mRNA of PLTP is ubiquitously expressed in various tissues with the highest levels found in lung, ovary, thymus, liver, adipose tissue, and placenta (*13*, *14*). The cDNA of PLTP encodes for a predicted 54-kDa protein, while the apparent molecular weight on SDS−PAGE gel is about 80 kDa, with the difference being due to glycosylation (*15*). In the circulation, PLTP exists in a high-activity (HA) form that binds preferentially apolipoprotein E and in a low-activity (LA) form that mainly interacts with apolipoprotein A-I (*16*, *17*). This may reflect the metabolic functions of PLTP (*18*), although the roles of HA-PLTP and LA-PLTP in lipoprotein metabolism remain unclear. In plasma, PLTP accounts for most of the phospholipid transfer activity (*19*, *20*). However, some phospholipid transfer activity is also due to CETP (*21*). The molecular mechanisms by which PLTP and CETP mediate lipid transfer are not completely resolved.

The ubiquitous expression of PLTP and CETP suggests that in addition to their role in lipoprotein metabolism they also have other functions and may play a role in lipid trafficking in human tear fluid and protect the corneal epithelium from excess lipid accumulation in a similar manner as tear lipocalin (*22*). The only reported lipid-binding protein in tear fluid is lipocalin. However, it is unclear whether lipocalin shows any lipid transfer activity and whether it is capable of binding new lipid molecules to its surface. The high lipid-binding capacity of PLTP (*23*) would make this protein well-suited for lipid transfer. However, because PLTP cannot transfer cholesteryl esters and triglycerides, we hypothesized that also CETP may be necessary for the maintenance of the neutral lipid content of the superficial tear film layer (*3*, *4*).

To gain insight into the mechanism of tear film formation, we have studied the occurrence of the two lipid transfer proteins PLTP and CETP in human tear fluid. We demonstrate that human tear fluid contains PLTP but not CETP. Analysis of tear fluid by size-exclusion and heparin-affinity chromatography indicates that tear fluid contains only a high active form of PLTP, representative of that found in plasma. These data indicate that human tear fluid shows significant PLTP activity, an observation that suggests a novel function for PLTP in lipid transfer processes in tear fluid.

EXPERIMENTAL PROCEDURES

Human Subjects. This study was performed according to the principles of the Declaration of Helsinki, and approved by the Ethics Review Committee of the Department of Ophthalmology (University of Helsinki). Informed consent was obtained from each subject. Tear fluid and plasma from 12 healthy subjects were used for the study. Clinical investigation of the subjects before collection of tear fluid showed no signs of ocular inflammation or allergy.

Tear Fluid Sample: Individual Samples. Tear fluid samples (approximately 20 μL) were collected from the lower conjunctival sac using Blaubrand intramark 10 μL micropipets (Brand GMBH, Wertheim, Germany) as reported (*24*). The samples were immediately cooled to +4 °C and stored at −70 °C until analyzed.

Tear Fluid Sample: Pooled Samples. To obtain large amounts of tear fluid, we stimulated tear production by onion vapor. These samples were collected from 11 subjects and combined for chromatographic analysis.

Blood Samples. Blood samples were collected into sterile heparin-containing tubes. The samples were kept at room temperature for 15 min followed by centrifugation at 3000*g* for 15 min at +4 °C. The plasma was separated and stored at −70 °C until used for analysis.

Antibodies. Mono- and polyclonal antibodies against PLTP were produced and purified as described (*25*). A monoclonal antibody against human CETP, TP-2, was a kind gift from Dr. Yves Marcel, Ottawa, Canada. Polyclonal antibodies against human apoA-I and apoE were raised in New Zealand white rabbits using antigens purified from human plasma.

Immunoinhibition of PLTP Activity. Increasing amounts of rabbit polyclonal anti-PLTP IgG (80−1280 μg) were added to human tear fluid (5 μL) and incubated for 2 h at room temperature. After volume adjustments, 20 μL aliquots were used to analyze PLTP activity. Control incubations contained PBS instead of anti-PLTP IgG.

PLTP, CETP, ApoA-I, and ApoE Immunoblotting. Western blot analysis was carried out as described (*26*).

Tear Lipocalin. Purified tear lipocalin was a kind gift from Dr. B. Redl (University of Innsbruck, Austria).

Measurement of PLTP Concentration and Activity. The concentration of PLTP in tear fluid and plasma was determined using an enzyme-linked immunosorbent assay (ELISA) (*18*). PLTP activity was recorded by measuring the transfer of ^{14}C-PC from radiolabeled PC vesicles to unlabeled HDL_3 acceptors (*27*), with minor modifications (*7*).

Cholesterol Ester Transfer Activity. Tear fluid CETP activity was determined from the rate of transfer of radiolabeled cholesterol ester from LDL to HDL (*28*).

Size-Exclusion Chromatography of Tear Fluid Proteins. Tear fluid combined from 11 subjects (500 μL) was subjected to a fast-performance liquid chromatography on a Superose 6 HR (10 × 30 cm) size-exclusion column (Amersham Pharmacia Biotech). The column was equilibrated with PBS (150 mM NaCl and 10 mM Na-phosphate at pH 7.4) containing 0.05% Tween 20. Chromatography was performed at a flow rate of 0.5 mL/min, and 0.5 mL fractions were collected for PLTP activity and mass measurements. The column was calibrated using protein standards (BioRad, Richmond, VA) having molecular weights between 1.35 and 670 kDa.

Heparin−Sepharose Chromatography of Tear Fluid Proteins. Tear fluid combined from 11 subjects (70 μL) was mixed with 930 μL of buffer A (25 mM Tris-HCl at pH 7.4 containing 1 mM EDTA) and applied on 1 mL Hi-Trap Heparin−Sepharose column (Amersham Pharmacia Biotech) pre-equilibrated with buffer A. After elution of nonbound material, buffer B (buffer A supplemented with 0.5 M NaCl) was applied and 0.5 mL fractions were collected at a flow rate of 0.5 mL/min. After buffer B, the elution was continued with buffer C (buffer A supplemented with 1 M NaCl). Collected fractions were analyzed for PLTP concentration and activity.

Quantification of ApoA-I and ApoE. ApoA-I and apoE in tear fluid samples were quantified by ELISA assays as described (*17*, *18*). ApoA-I and apoE were also determined by Western blot analysis with specific anti-human apoA-I

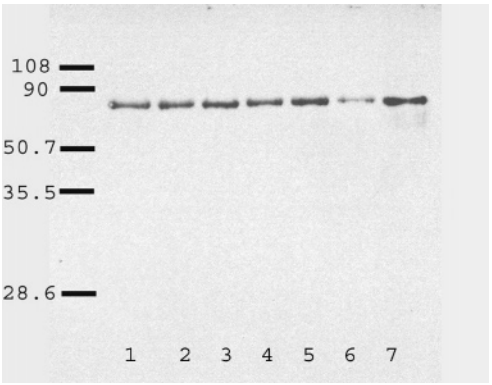

FIGURE 1: Western blot analysis of PLTP in tear fluid and plasma. Lanes 1−7, tear fluid of healthy subjects. Positions of molecular-weight markers (in kilodaltons) are indicated. Immunoblotting was performed using a monoclonal human anti-PLTP antibody.

and apoE antibodies and ECL, electrochemiluminescence detection (*26*).

Statistical Analysis. Results are expressed as the mean ± standard deviation. The densitometric and lipid transfer assay values were analyzed by Student's *t* test. A *p* value of less than 0.05 was considered statistically significant. Pearson's correlation coefficients were used for evaluation of parametric correlations.

RESULTS

Detection of PLTP in Human Tear Fluid. Western blotting of human tear fluid and plasma using a monoclonal anti-PLTP antibody (*25*) revealed that both tear fluid and plasma displayed a single ~80-kDa band corresponding to the predicted molecular weight of the mature, highly glycosylated form of human PLTP (Figure 1).

The concentration of PLTP in tear fluid and plasma was determined using ELISA for human PLTP (*18*). The mean PLTP concentration in tear fluid was 10.9 ± 2.4 μg/mL and in plasma, 4.5 ± 0.9 μg/mL ($p < 0.001$); Figure 2A. Thus, the PLTP concentration seems to be higher in tear fluid than in plasma. The mean phospholipid transfer activity in tear fluid was 15.1 ± 1.8 μmol mL^{-1} h^{-1} and in plasma, 8.9 ± 1.8 μmol mL^{-1} h^{-1} ($p < 0.001$) (Figure 2B). The specific activity of PLTP in tear fluid compared to plasma was found to be significantly lower (1.4 ± 0.3 nmol μg^{-1} h^{-1} versus 2.1 ± 0.6 nmol μg^{-1} h^{-1}, $p = 0.006$; Figure 2C). No correlation between tear and plasma PLTP concentration and activity was observed (data not shown).

The Contribution of PLTP to Phospholipid Transfer Activity in Tear Fluid. To determine how much PLTP contributed to the total phospholipid transfer activity in tear fluid, we studied the effect of inhibiting PLTP by heat treatment (58 °C for 60 min) or by a monospecific polyclonal anti-PLTP antibody. Heat inactivation of PLTP (*29*) resulted in the loss of transfer activity (data not shown). In addition, immunoinhibition with a polyclonal antibody specific for PLTP resulted in almost total inhibition of PLTP activity (Figure 3).

Finally, we used purified tear lipocalin in the lipid transfer activity measurements instead of tear fluid. Tear lipocalin did not show phospholipid transfer activity in the assay developed to measure PLTP-facilitated phospholipid transfer (data not shown).

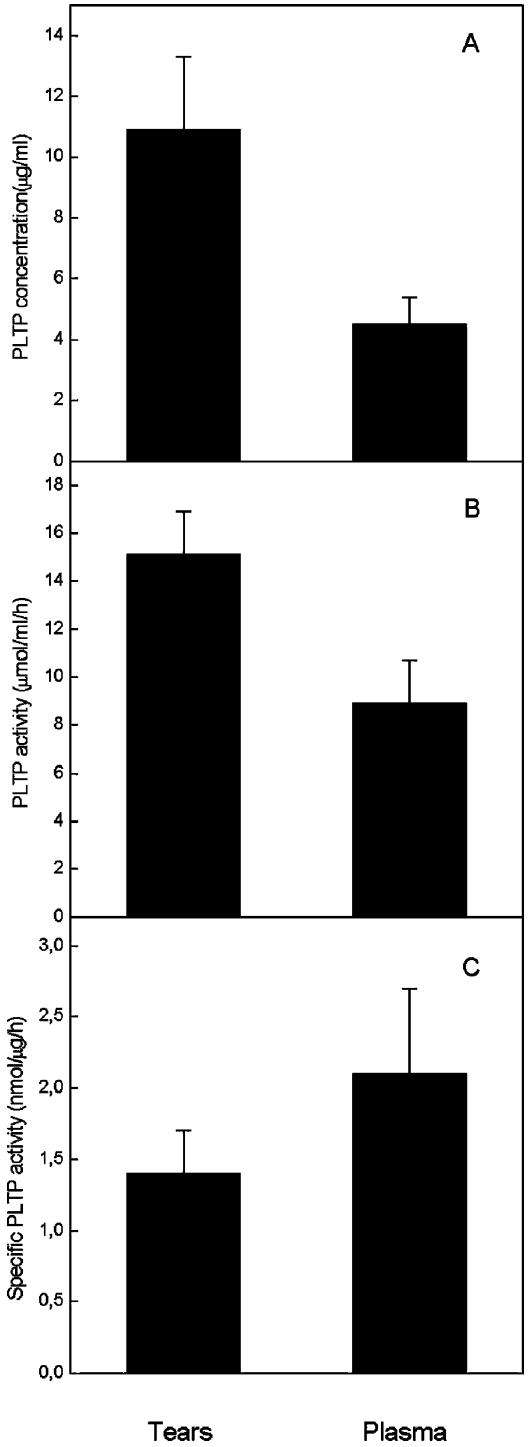

FIGURE 2: PLTP concentration (A), activity (B), and specific activity (C) in tear fluid and plasma. PLTP concentration was determined by specific ELISA as described in the Experimental Procedures. PLTP activity was obtained by measuring the transfer of ^{14}C-PC from radiolabeled liposomes to unlabeled HDL_3 acceptors. Specific phospholipid transfer activity (nmol μg^{-1} h^{-1}) is calculated by dividing the phospholipid transfer activity by the PLTP concentration.

Characterization of Tear PLTP. Catalytically active plasma PLTP can be separated from the catalytically low-activity form by size-exclusion chromatography or by using the heparin-binding properties of the active protein (*16*, *17*). When analyzed by size-exclusion chromatography (Figure 5A, OD_{280} curve), PLTP appeared to be only a minor component among the tear fluid proteins. PLTP activity and

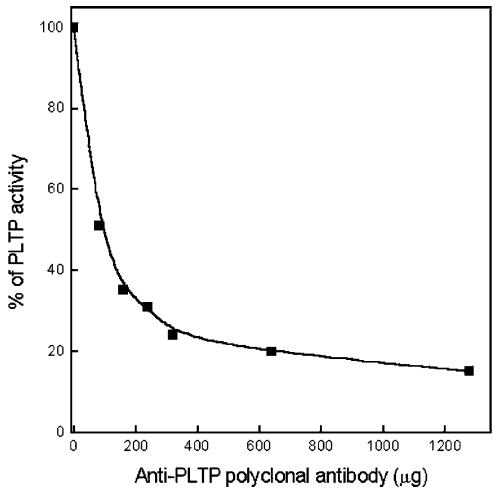

FIGURE 3: Immunoinhibition of PLTP with a polyclonal anti-PLTP antibody. Increasing amounts of rabbit polyclonal anti-PLTP IgG (80−1280 μg) were added to human tear fluid (5 μL) and incubated for 2 h at room temperature. After volume adjustments, 20 μL aliquots were used to analyze PLTP activity. Control incubations contained PBS. Phospholipid transfer activity was determined as described in the Experimental Procedures. The *y* axis presents PLTP activity as a percentage of the control value, and the *x* axis displays anti-PLTP IgG. The 100% value of PLTP activity was 11 600 nmol h^{-1} mL^{-1}.

PLTP protein coeluted in fractions 29−31, corresponding to an apparent molecular weight of 160−170 kDa (Figure 4B and inset). Catalytically inactive PLTP was not observed in any of the fractions. During Heparin−Sepharose-affinity chromatography, both PLTP activity and mass bound to the column and could be eluted with 0.5 M NaCl (Figure 5). The recovery of PLTP activity was over 90% and that of PLTP protein mass was about 80%. These results demonstrate that PLTP in tear fluid has similar characteristics to the high-activity form of PLTP present in plasma.

Apolipoproteins A-I and E in Human Tear Fluid. In human plasma, LA-PLTP is found associated with apolipoprotein (apo) A-I, while HA-PLTP associates preferentially with apoE (*17*, *30*). This suggests that PLTP activity may be regulated by the apoE/apoA-I molar ratio in the PLTP−apolipoprotein complex. Because PLTP is highly hydrophobic, we reasoned that PLTP might be associated with these apolipoproteins also in tear fluid. We analyzed tear fluid for the presence of these apolipoproteins using Western blotting and ELISA. However, neither apoA-I nor apoE could be detected (data not shown).

CETP in Tear Fluid. By Western blotting using a monoclonal antibody, no immunoreactive CETP could be detected in tear fluid (data not shown). The presence of other possible neutral lipid transferring activities in tear fluid was studied by an isotopic assay, which measures transfer of radioactive cholesteryl ester from LDL to HDL_3. Consistent with the lack of immunoreactive CETP, no cholesteryl ester transfer activity could be detected in tear fluid (data not shown). PLTP and CETP can be separated by Heparin−Sepharose-affinity chromatography (*29*), whereby PLTP is quantitatively bound to heparin, while CETP is not. In the flow-through fractions of Heparin−Sepharose chromatography, we could not detect either CETP activity or immunodetectable CETP. These observations strongly suggest that

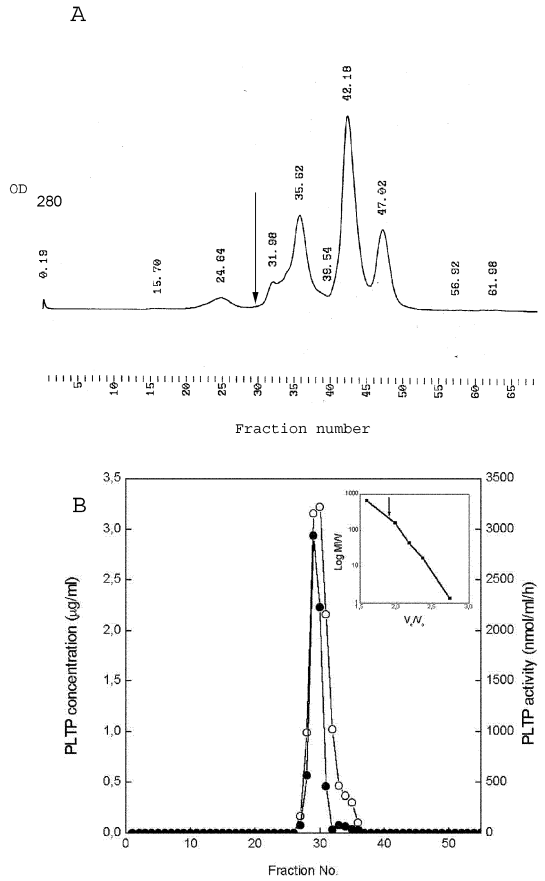

FIGURE 4: Size-exclusion chromatography of human tear fluid. Pooled human tear fluid (500 μL) was injected on a Superose 6 HR 10/30 column. Elution was performed at a 0.5 mL/min flow rate with 10 mM Na-phosphate and 150 mM NaCl at pH 7.4 containing 0.05% Tween 20. Fractions (0.5 mL each) were collected and analyzed for total protein as the optical density at 280 nm (A). The arrow in A depicts the position where the highest PLTP activity and mass were eluted. PLTP activity (○) as well as PLTP concentration (●) in the same fractions are presented in B. (Inset) Determination of apparent molecular weight of the eluted PLTP during size-exclusion chromatography using the correlation between the relative elution volume (V_e/V_o) and the molecular weight of proteins. V_e, elution volume of the protein; V_o, outer volume of the matrix; V_e/V_o, relative elution volume. The arrow depicts the V_e/V_o for PLTP.

tear fluid does not contain CETP. Purified tear lipocalin had no neutral lipid transferring activity (data not shown).

DISCUSSION

Our study reports for the first time that human tear fluid displays phospholipid transfer activity. PLTP activity as well as PLTP protein were observed in all tear samples studied, suggesting that it is a normal component of the tear fluid and may play a role in housekeeping protein machinery. In contrast to the circulation where PLTP is detected in two forms, one catalytically active and the other with low activity, tear fluid contained only a high active form of PLTP (HA-PLTP). Although the concentration and activity of PLTP was significantly higher in tear fluid compared to plasma, the specific activity of this enzyme was found to be significantly lower in tear fluid. Because no correlation between tear and plasma PLTP concentration, activity, or specific activity was observed, it is likely that tear fluid PLTP does not originate from circulation. Accordingly, it seems plausible

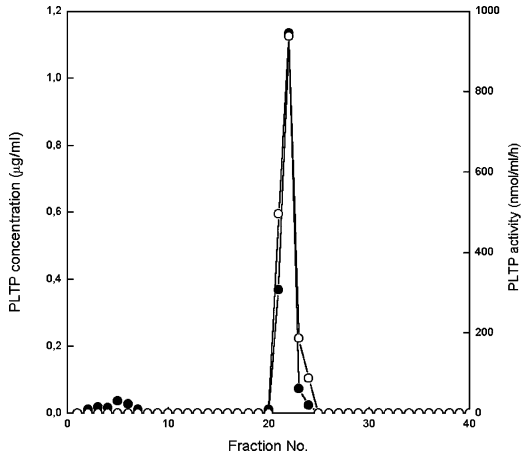

FIGURE 5: Heparin−Sepharose (H−S) affinity chromatography of tear fluid. A total tear volume of 70 μL in 930 μL of 25 mM Tris-HCl at pH 7.4 containing 1 mM EDTA was applied to a 1 mL HiTrap H−S column. The column was first washed with 25 mM Tris-HCl buffer at pH 7.4 and 1 mM EDTA to remove nonbound material after which heparin-bound material was eluted with 0.5 M NaCl. Fractions were analyzed for PLTP activity (○) and concentration (●).

that tear PLTP is most likely secreted either form the lacrimal gland, corneal epithelium, or conjunctival epithelium.

In addition to PLTP, also other tear proteins, such as tear lipocalin, might mediate lipid transfer in tear fluid. Accordingly, immunoprecipitation and heat inactivation of PLTP were used to determine the contribution of tear PLTP in lipid transfer. Results when using these two methods demonstrate that the majority of the phospholipid transfer activity was facilitated via PLTP function. Cholesteryl ester transfer activity was not detected in tear fluid. Finally, we found no evidence that tear lipocalin would transport either polar or neutral lipids in tear fluid using the lipid transfer assays. The physiological function of PLTP in tear fluid is yet to be discovered.

The finding that the specific activity of tear PLTP was lower compared to plasma implicates that enzyme activity could be differently regulated. It may be that the proteins have different sets of oligosaccharide structures, and possibly their different specific activities might reflect these variations. Another possibility for the difference in specific activity is that the activity of plasma PLTP is partly regulated by protein−protein interactions. During purification of plasma PLTP, apoA-I associates with the low-activity form of PLTP, whereas the high-activity form preferentially interacts with apoE (*17*, *30*). As a first step to identifying the proteins that tear PLTP might associate, we analyzed the presence of apoA-I and apoE in tear fluid. Neither apoA-I nor apoE were found in tear fluid, suggesting that the observed lowered specific activity might reflect the absence of these molecules. We are currently aiming to identify the possible proteins that form protein−protein complexes with PLTP in tears. It remains possible that in tear fluid no protein will be found associated with PLTP and that PLTP is still catalytically active. For example, during our normal PLTP purification process from plasma, the final purified PLTP protein does not contain any other copurified proteins and is nevertheless highly active (*7*). This is further supported by the observations that human PLTP produced in the baculovirus protein expression system (*18*) as well as PLTP in human seminal plasma (*31*) are fully active but neither contain apoA-I nor apoE. One plausible explanation for this is that PLTP binds phospholipids maintaining the active conformation. This concept is supported by the findings that purified plasma PLTP indeed interacts with several phospholipid molecules (*23*). These observations might suggest a possible different physiological function of PLTP in tear fluid compared to plasma.

PLTP in human tear fluid displayed characteristics very similar to the high activity form in human plasma (*16*, *17*). First, PLTP activity and protein comigrated in a position corresponding to the apparent molecular mass of 160−170 kDa during size-exclusion chromatography, and second, PLTP activity and mass were quantitatively bound on a Heparin−Sepharose column. This suggests that the tear fluid compartment is lacking the molecular machinery that facilitates the generation of the low-activity form of PLTP observed in plasma. It remains to be established if the two forms of PLTP possess different functions in plasma or if LA-PLTP is just a remnant of HA-PLTP. As anticipated previously, LA- and HA-PLTP binding to lipids needs to be studied and may give a clue as to whether lipid composition is related to the activity of PLTP in tear fluid.

One explanation for why CETP was not detected in human tear fluid is that CETP may not reach cholesteryl esters/triglycerides in the superficial lipid layer or that there is no need for such enzyme activity. More specifically and conventionally, the superficial tear film is composed of 2−3 lipid layers. Phospholipids and other amphiphilic molecules are located next to the aqueous interface because of the hydrophobic effect (*32*). Externally to this, cholesteryl esters/triglycerides are located on a more disordered manner in 1−2 layers. Accordingly, phospholipids cause a steric constraint for CETP or other neutral lipids transferring protein to reach their ligands. The evidence for the presence of cholesterol esters and triglycerides in tear fluid derives from chemical analysis of the composition of the meibomian gland excretions (*4*, *33*). *A priori*, we find no reason that these neutral lipids should be transported to the superficial lipid layer. Therefore, we analyzed the lipid composition of the tear fluid with enzymatic assays. The major portion of lipids were phospholipids, whereas the concentration of cholesterol and neutral lipids was below the detection limit (unpublished data). This scenario would provide a very convenient explanation for the lack of cholesteryl ester transfer activity in tear fluid and would necessitate a revision of the arrangement of the superficial tear film lipid layer.

The widespread distribution of PLTP mRNA in peripheral tissues suggests that PLTP may play a significant role in regulating extracellular phospholipid and lipoprotein metabolism (*11*, *34*). It is interesting to note that the amount of lung surfactant among cigarette smokers is reduced (*35*), and possibly as a counterbalancing effect, the number of epithelial type-II alveolar cells is increased. Likewise, among patients suffering from emphysema because of smoking, PLTP is highly overexpressed (*14*). Because epithelial type-II alveolar cells in the lung secrete PLTP, it is possible that hyperplasia could stimulate PLTP production to transport more surfactant to the damaged alveolar surfactant membrane. A somewhat analogous situation to emphysematous lungs might be what takes place in dry eye syndrome or KC. More specifically, the superficial lipid layer provides a barrier to prevent

evaporation of tear fluid that would eventually lead to pathological damage of the corneal epithelium. In tear fluid, PLTP could facilitate the uptake of meibomian lipids and ultimately transport them to the superficial lipid layer. Likewise, if the corneal epithelium would be contaminated by hydrophobic material, PLTP could scavenge these and transport them to the superficial layer of the tear fluid or, alternatively, scavenge lipids by binding them and transporting them through the naso-lacrimal duct. PLTP might play an integral role in tear lipid trafficking and in the protection of the corneal epithelium.

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BI050151K