Plasma cholesteryl ester transfer protein and blood coagulability

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Summary
Dyslipoproteinemia involving low levels of high density lipoprotein (HDL) is linked to venous thrombosis in young male adults and to recurrence of venous thrombosis in patients who have experienced a previous unprovoked venous thrombosis episode. Plasma cholesteryl ester transfer protein (CETP) modulates HDL metabolism and some lipoproteins can affect blood coagulation reactions with either procoagulant or anticoagulant effects. Hence, we evaluated relationships between the mass of CETP and blood coagulability in plasma samples from 39 normal healthy adults. For clotting initiated by dilute tissue factor or factor XIa, clotting times significantly correlated with CETP antigen levels. Thus, coagulation initiated by either the extrinsic or intrinsic coagulation pathway is positively correlated with CETP plasma levels. When added to plasma, a recombinant CETP preparation dose-dependently shortened factor Xa-1-stage clotting times, showing that it augmented procoagulant activity in plasma. In reaction mixtures containing purified factors Xa and Va and prothrombin, the recombinant CETP preparation dose-dependently increased prothrombin activation, suggesting it specifically enhances prothrombinase activity. Thus, our data highlight a previously unknown positive relationship between CETP plasma levels and blood coagulability that might relate to risks for thrombotic events.

Keywords
CETP, coagulation factors, thrombosis, HDL

Introduction
Venous thromboembolic disease (VTE) is associated with an increase in coagulability and is a polygenic disease with pathogenic contributions from both genetic and environmental risk factors (1, 2), and several studies of plasma or serum lipids have shown an association between dyslipidemia and VTE (3–7). Lipoproteins are the major carriers of plasma lipids and lipoprotein particles manifest multiple biologic activities. We found that dyslipoproteinemia, especially high density lipoprotein (HDL) deficiency, is associated with the occurrence of VTE in males under 55 years old (8) and with the recurrence of VTE in adults after one episode of unprovoked VTE (9). Plasma cholesteryl ester transfer protein (CETP) is one of the key modulators of lipoprotein metabolism. The common TaqI B1 variation in the CETP gene was linked to VTE in young males (8) as were two relatively rare CETP variations, Ala373 to Pro and Arg451 to Gln, that cause elevated plasma CETP and an unfavorable pattern of lipoproteins (10). Although some blood clotting reactions are directly affected by lipoprotein particles (11, 12) and HDL has multiple antithrombotic properties (13), there is currently no information available which addresses the potential relationships between plasma CETP, a key modulator of lipoprotein metabolism, especially HDL metabolism (14), and blood coagulation reactions. Here we describe for the first time notable relationships between CETP and blood coagulability.

Materials and methods

Study group
Thirty-nine healthy volunteers (19 male, 20 female) were recruited through the General Clinical Research Center’s (GCRC) blood donation program at The Scripps Research Institute (35.8 ± 7.1 years old, range: 19–56). These healthy laboratory blood donors were routine volunteers who were not taking oral contraceptives or estrogen replacement therapy or any medications. The major thrombophilic risk factors of factor V Leiden and prothrombin 20210A were not found in these healthy volunteers. Informed consent was obtained from each subject in accordance with the Declaration of Helsinki. Blood samples were obtained...
from routine vein puncture after an overnight fast then mixed with 0.129 M sodium citrate (9:1). Plasma was prepared by centrifugation at 2,000 x g for 20 min at room temperature and then stored at −80°C. Clinical characteristics, the frequency of identified risk factors and serum lipid data are shown in Table 1.

Coagulation assays
Clotting of diluted plasma samples supplemented with fibrinogen was induced by dilute tissue factor (1:40 of Innovin, 350 pM final tissue factor) (11), or similarly by the addition of purified factor Xa (7.5 nM, final) to test the coagulability of individual samples. The between-run precision was assessed by repeated measurements (n=10) for dilute tissue factor or factor Xa induced clotting assay using pooled plasma (George King Biomedical, INC, Overland Park, KA) (coefficient of variation=2.3% and 1.1%, respectively). Factor Xa (0.9 nM, final) 1-stage assays were used to study exogenously added CETP. For these clotting assays, the amount of exogenously added phospholipid was not significant that the assays were sensitive to endogenous plasma lipids and lipoproteins (15). In contrast, activated partial thromboplastin time (APTT) assays (Platelin LS) contain excess exogenous phospholipids and are not very sensitive to endogenous plasma lipoproteins or lipids.

Measurements of plasma lipids/lipoproteins and proteins
CETP antigen was measured by ELISA (Wako Chemicals, Inc, Richmond, VA, USA). Coagulation factor antigen levels were measured using commercial immunoassay kits. Choline-containing phospholipids (mainly phosphatidylcholine and sphingomyelin) were quantified by enzymatic assay kit (Wako Chemicals, Inc, Richmond, VA, USA). Glucosylerceramide, cardiolipin, phosphatidylserine and phosphatidylethanolamine in plasma were measured using HPLC (15). Triglyceride, total cholesterol, HDL cholesterol and low density lipoprotein (LDL)-cholesterol were measured by proton NMR spectroscopy (Liposcience, Raleigh, NC, USA). Recombinant human CETP preparations (rCETP) were purchased from Cardiovascular Targets Inc (New York, NY, USA). Clotting factors were purchased from Haematologic Technologies (Essex Junction, VT, USA).

Prothrombin activation assays
Prothrombin (0.76 µM final) activation by purified factors Xa (0.7 nM final) and Va (varying final concentrations) plus or minus varying concentrations of rCETP preparation was assayed in the absence of exogenously added phospholipids. Reactants were mixed and incubated at room temperature for 5 min to allow prothrombin activation before the reaction was quenched by EDTA, and then the amidolytic activity using the Pefachrome TH thrombin (IIa) chromogenic substrate was quantified. The rate of prothrombin activation was then calculated in units of nM thrombin formed per min (IIa nM/min).

Statistical analysis
Pearson’s correlation analyses were made using GraphPad Prism 4.0 (GraphPad Software for Science Inc., San Diego, CA, USA). MINITAB14 software (Minitab Inc., State College PA, USA) was used to determine partial Pearson’s correlation coefficient.

Table 1: Study population characteristics (one SD).

<table>
<thead>
<tr>
<th>Variables</th>
<th>N=39</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, yr (SD)</td>
<td>35.8 (7.2)</td>
</tr>
<tr>
<td>Gender, female (%)</td>
<td>20 (51.3)</td>
</tr>
<tr>
<td>Ethnic Group</td>
<td></td>
</tr>
<tr>
<td>Non-Hispanic White, %</td>
<td>61.5</td>
</tr>
<tr>
<td>CETP Ag, µg/ml (SD)</td>
<td>1.18 (0.29)</td>
</tr>
<tr>
<td>Lipids (NMR), mean (SD)</td>
<td></td>
</tr>
<tr>
<td>total cholesterol, mg/dl</td>
<td>154.7 (30.6)</td>
</tr>
<tr>
<td>Triglyceride, mg/dl</td>
<td>108.9 (57.4)</td>
</tr>
<tr>
<td>HDL-C, mg/dl</td>
<td>43.5 (10.4)</td>
</tr>
<tr>
<td>LDL-C, mg/dl</td>
<td>92.5 (23.6)</td>
</tr>
</tbody>
</table>

Stepwise linear regression analysis was applied to determine standardized coefficient using MINITAB. Statistical significance was achieved when two-tailed p values were <0.05.

Results and discussion
In order to determine whether endogenous plasma CETP antigen levels are linked to coagulability, samples from 39 healthy adults were assayed using modified PT (11) or factor Xa-1-stage clotting assays that reflect the extrinsic or intrinsic coagulation pathways, respectively. There was a significant inverse correlation of plasma CETP antigen levels with clotting times induced by dilute tissue factor (r =−0.43, p=0.007) and by factor Xa (r =−0.35, p=0.03) (Table 2, Fig. 1a, b). When the clotting data were stratified by gender, the inverse correlations of plasma CETP antigen levels with clotting times induced by dilute tissue factor and by factor Xa for the male subgroup remained statistically significant (r =−0.61, p=0.006 and r =−0.45, p=0.05, respectively) but not for the female subgroup (r =−0.16, p=0.49 and r =−0.21, p=0.36, respectively). Interestingly, gender is an important variable for VTE as male gender compared with female gender is an independent risk factor for VTE recurrence (16, 17).

Tissue factor-induced clotting times also correlated with plasma levels of total phospholipid (r =−0.45, p=0.004), total cholesterol (r =−0.43, p=0.01), total glucosylerceramide (r =−0.39, p=0.02) and coagulation factor V antigen (r =−0.46, p=0.003) (Table 2, Fig. 1). However, similar associations were not observed for factor Xa-induced clotting. APTT assays with excess exogenously added phospholipids did not show any correlation with plasma CETP levels (Table 2). Tissue factor-induced clotting showed an inverse association with CETP antigen plasma levels even after adjustment for total cholesterol, phospholipid, total glucosylerceramide and factor V (Pearson partial correlation coefficient r =−0.41, p=0.01); furthermore, the inverse association with CETP antigen level remained even after adjustment for HDL-cholesterol which CETP can modulate (Pearson partial correlation coefficient r =−0.41, p=0.01). Thus, the CETP inverse correlation with clotting times was independent of these analytes.

The independence of CETP antigen, phospholipids, GlcCer, total cholesterol and factor V for predicting tissue factor-induced...
clotting times was further analyzed based on stepwise multiple regression analysis. Four of these analytes, namely CETP, phospholipids, GlcCera and factor V, remained independent predictors of dilute tissue factor-induced clotting times based on values for standardized coefficients of –0.32, –0.24, –0.27, and –0.28, with p values of 0.003, 0.02, 0.01 and 0.02, respectively. The correlation of total cholesterol with tissue factor-induced clotting times lost significance after this stepwise multiple regression analysis. Among the variables analyzed, CETP antigen levels showed the strongest independent relationship to clotting induced by tissue factor or factor XIa; and CETP, phospholipids, glucosylceramide and factor V were each independently related to clotting in these assays (Table 2). For clotting initiated by dilute tissue factor or factor XIa without the addition of exogenous lipids, there was no statistically significant correlation with HDL-cholesterol, LDL-cholesterol, triglycerides, cardiolipin, phosphatidylserine, phosphatidylethanolamine, prothrombin antigen, factor Xa antigen or factor VIII antigen (Table 2), emphasizing the remarkable associations between CETP plasma levels and clotting induced by activators of both the extrinsic and intrinsic coagulation pathways.

What might be the basis for the association of CETP plasma levels with coagulability? Is it coincidental or possibly, in part, causal? CETP does directly influence lipoprotein metabolism and it can alter the plasma lipid profile; lipids affect clotting reactions, and this might relate to an indirect effect on the coagu-

Table 2: Correlations between blood coagulability determined by clotting assays and plasma CETP antigen levels and between CETP antigen levels and other plasma analytes.

<table>
<thead>
<tr>
<th>Clotting induced by</th>
<th>Tissue factor</th>
<th>Factor Xla</th>
<th>APTT</th>
<th>CETP antigen</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>r</td>
<td>p</td>
<td>r</td>
<td>p</td>
</tr>
<tr>
<td>CETP antigen</td>
<td>–0.43</td>
<td>0.007</td>
<td>–0.35</td>
<td>0.03</td>
</tr>
<tr>
<td>Total cholesterol</td>
<td>–0.43</td>
<td>0.01</td>
<td>–0.22</td>
<td>0.22</td>
</tr>
<tr>
<td>LDL-Cholesterol</td>
<td>–0.30</td>
<td>0.09</td>
<td>–0.13</td>
<td>0.48</td>
</tr>
<tr>
<td>HDL-Cholesterol</td>
<td>–0.31</td>
<td>0.08</td>
<td>–0.17</td>
<td>0.33</td>
</tr>
<tr>
<td>Triglyceride</td>
<td>–0.29</td>
<td>0.09</td>
<td>–0.16</td>
<td>0.37</td>
</tr>
</tbody>
</table>

Phospholipid 1 | –0.45 | 0.004 | –0.15 | 0.36 | –0.08 | 0.64 | 0.10 | 0.53 |
| Glucosylceramide | –0.39 | 0.02 | –0.03 | 0.86 | –0.33 | 0.04 | 0.09 | 0.60 |
| Cardiolipin | –0.20 | 0.22 | –0.23 | 0.16 | –0.38 | 0.02 | –0.03 | 0.85 |
| Phosphatidylserine | –0.04 | 0.79 | –0.02 | 0.91 | –0.08 | 0.64 | 0.27 | 0.09 |
| Phosphatidylethanolamine | –0.14 | 0.39 | 0.01 | 0.95 | –0.08 | 0.65 | –0.13 | 0.43 |
| Prothrombin antigen | –0.16 | 0.32 | –0.10 | 0.53 | –0.00 | 1.00 | 0.05 | 0.75 |
| FV antigen | –0.46 | 0.003 | 0.12 | 0.48 | 0.10 | 0.54 | –0.04 | 0.83 |
| Factor X antigen | –0.12 | 0.48 | 0.03 | 0.85 | –0.08 | 0.63 | 0.15 | 0.37 |
| FVIIa antigen | –0.12 | 0.48 | –0.26 | 0.11 | –0.35 | 0.03 | 0.23 | 0.16 |
| FVIII antigen | –0.01 | 0.93 | –0.16 | 0.34 | –0.36 | 0.02 | 0.28 | 0.09 |

Significant correlations are shown in bold font. CETP antigen (last column) was not statistically correlated with any of the indicated plasma analytes (p < 0.05). 1 Choline-containing phospholipids (mainly phosphatidylcholine and sphingomyelin).

Figure 1: Correlation between plasma CETP antigen and blood coagulability and between other plasma analytes and blood coagulability in healthy blood donors. Coagulability of fasting citrated plasma samples from 39 healthy donors (20 female, 19 male who gave informed consent) was assayed using either a modified prothrombin time assay (modified PT) in which clotting was induced by either diluted tissue factor (1:40 Innovin) or a factor Xla-1-stage assay (7.5 nM factor Xla). Correlations are shown for (a) modified PT assays with CETP antigen and (b) factor Xla induced clotting with CETP antigen. Pearson correlation coefficient (r) and p values are indicated.
lability. To explore potential direct effects of CETP, we investigated the influence of recombinant CETP preparations (rCETP) on coagulability. Because our data showed correlations between CETP antigen levels and two clotting assays that reflected both the extrinsic and intrinsic blood coagulation pathways (Table 2), we speculated that CETP might affect reactions that are common to both pathways, namely prothrombin activation (19). Thus, rCETP direct effects on factor Xa-induced clotting in plasma and on prothrombin activation in purified reaction mixtures were assayed. rCETP dose-dependently shortened the clotting time (Fig. 2a) and, in purified reaction mixtures, enhanced prothrombin activation by factors Xa and Va (Fig. 2b). Thus, rCETP appears to enhance prothrombin activation.

It is unclear how CETP can affect prothrombin activation by either direct or indirect mechanisms. CETP itself might interact with coagulation proteins to modulate their activity. On the other hand, lipids play key roles for the assembly and procoagulant functioning of clotting factor complexes, and CETP has lipid transfer activities. Crystallized CETP contains two cholesteryl ester molecules and two phospholipid molecules that remarkably are sequestered in a 60 Å long hydrophobic tunnel which possesses two openings on the CETP surface (19). Hence, we speculate that CETP might exchange a variety of lipids that promote prothrombin activation and perhaps other clotting reactions. Further studies of interactions among rCETP, prothrombin, factors Xa and Va and procoagulant lipids are needed to decipher the molecular interactions that underlie rCETP procoagulant effects.

Reports that HDL-cholesterol is increased by either inhibition of plasma CETP or CETP genetic deficiency implied that pharmacologic inhibition of CETP would decrease atherosclerotic burden and cardiovascular events in patients at high risk and led to clinical trials of a new class of potential drugs, inhibitors of CETP (20–22). Although torcetrapib did raise HDL-C level, recent trials reported no benefit of the CETP inhibitor, torcetrapib, on coronary atherosclerosis or carotid artery intimal medial thickness (23–25). Furthermore, the large phase III ILLUMINATE trial of torcetrapib in combination with atorvastatin versus atorvastatin alone was halted due to excess deaths in the torcetrapib treated group vs. controls (82 deaths versus 51 deaths) (23, 26, 27). Currently, it is unclear whether torcetrapib’s excess mortality is related to its alterations of HDL or whether it is entirely independent of the drug’s effects on HDL (23, 26). Although the CETP inhibitors, torcetrapib and JTT-705, inhibit the cholesteryl ester transfer activity of CETP and raise HDL-cholesterol levels, they also actually raise the plasma CETP antigen level up to 3.5-fold, apparently because the CETP inhibitor complex binds more avidly to HDL than CETP alone, thus prolonging CETP’s half-life (20–22, 27). Our findings that blood coagulability is positively correlated with CETP antigen and that rCETP promotes prothrombin activation might provide a possible explanation for the discrepancy between the remarkable increase of HDL-cholesterol induced by torcetrapib and the negative outcome of the ILLUMINATE torcetrapib trial (23, 26). One might hypothesize that the increase of CETP antigen by torcetrapib can increase blood coagulability with a negative influence on pro-atherosclerotic or prothrombotic processes. Further experimentation is necessary in order to explore this hypothesis.

In summary, our data show that CETP in plasma is positively associated with blood coagulability in normal healthy adults, especially in men, and this discovery raises a variety of interesting mechanistic and clinical questions. rCETP appears capable of promoting clotting in plasma, in part by enhancing prothrombinase activity, and further work on mechanisms for this phenomenon is warranted. Torcetrapib and JTT-705 raise plasma CETP antigen levels (20–22, 27). Thus, the obvious question arises of whether CETP inhibitor treatment might directly or indirectly increase coagulability of blood and thereby increase the risk for thrombotic events. Appropriately designed preclinical and clinical studies are needed to address this and related questions.

Acknowledgement

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