Chapter 8

REDUCED LEUKOCYTE CHOLESTERYL ESTER TRANSFER PROTEIN EXPRESSION IN ACUTE CORONARY SYNDROMES

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ABSTRACT

Cholesterol ester transfer protein (CETP) plays an important role in HDL cholesterol metabolism. Leukocytes, including monocyte-derived macrophages in the arterial wall synthesize and secrete CETP, but its role in atherosclerosis is unclear. The aim of the current study was to investigate the effect of acute coronary syndromes (ACS) on leukocyte CETP expression. Peripheral blood mononuclear cells (PBMCs) were freshly isolated from hospitalized ACS patients displaying Braunwald class IIIB unstable angina pectoris (UAP) on admission (t=0) and at 180 days post inclusion (t=180) for analysis of CETP expression. In addition, to prove the potential correlation between leukocyte CETP and acute coronary syndromes the effect of acute myocardial infarction on leukocyte CETP expression was studied in CETP transgenic mice. Upon admission, UAP patients displayed ~3-6-fold (p<0.01) lower CETP mRNA and nearly absent CETP protein expression in PBMCs, as compared to healthy age/sex matched controls. Interestingly, CETP mRNA and protein levels were significantly elevated in PBMCs isolated from UAP patients (both stabilized and refractory) at t=180 as compared to t=0 (p<0.01), which was correlated with a reduced inflammatory status after
medical treatment. In agreement with the data obtained in UAP patients, markedly down-regulated leukocyte CETP mRNA expression was observed after coronary artery ligation in CETP transgenic mice, which also correlated with increased serum amyloid A levels.

We are the first to report that episodes of UAP in humans and myocardial infarction in CETP transgenic mice are associated with reduced leukocyte CETP expression. We propose that the impairment in leukocyte CETP production is associated with an enhanced inflammatory status, which could be clinically relevant for the pathogenesis of ACS.

INTRODUCTION

Prospective epidemiological studies have clearly shown an inverse correlation between high-density lipoprotein (HDL) and the incidence of coronary artery disease (CAD).\(^1\)\(^2\) Cholesteryl ester transfer protein (CETP) promotes the transport of cholesteryl ester (CE) from anti-atherogenic HDL to pro-atherogenic apoB-containing particles in exchange for triglycerides (TGs).\(^3\) As such, CETP plays a crucial role in HDL metabolism. However, the relationship between CETP, HDL, and the risk of CAD remains controversial.

Transgenic mice expressing human CETP (CETP Tg) show reduced HDL cholesterol (HDL-C) levels, increased apoB-containing lipoproteins, and an enhanced susceptibility to atherosclerosis as compared to wild-type controls.\(^4\)\(^5\) In men with CAD, high plasma CETP concentrations are strongly associated with a faster progression of atherosclerosis in the REGRESS study.\(^6\) Moreover, homozygous genetic deficiency of CETP in humans is associated with marked elevations in HDL-C and a reduced prevalence of CAD.\(^7\) However, others have reported that CETP deficiency increases the CAD risk despite elevated HDL levels.\(^8\)\(^9\) No significant contribution of the plasma CETP concentration in determining HDL-C has been observed in normolipidaemic healthy subjects to date.\(^10\)\(^11\) Furthermore, plasma CETP is suggested to positively correlate with future CAD risk only in hypertriglyceridemic subjects.\(^12\) Actually, the ultimate effect of CETP on atherosclerosis might depend on the metabolic, genetic, and environmental context, as well as potentially differential effects of CETP on systemic lipoprotein metabolism and local conditions in the artery wall.

In vitro, human monocyte-derived macrophages synthesize and secrete CETP,\(^13\) and a direct role for macrophage CETP in cellular cholesterol efflux has been shown.\(^14\) CETP has been detected in various human tissues such as liver, adipose, spleen, and tissue macrophages, and the CETP protein is secreted to a variable extent from each of these tissues into plasma.\(^15\) Mice are naturally CETP deficient, but studies with transgenic animals expressing CETP have proven to be of help to further unravel the role of CETP in lipid metabolism.\(^15\) We recently showed that bone marrow (BM)-derived CETP is an important contributor to the total circulating CETP mass in mice by transplantation of BM from CETP Tg mice into LDL receptor knockout mice.\(^16\) Furthermore, specific disruption of CETP in BM-derived cells by transplantation of wild-type BM into CETP Tg mice indicated that 50% of the circulating CETP in these mice originates from BM-derived cells.\(^16\) CETP belongs to the family of lipid transfer/lipopolysaccharide-binding proteins. Lipopolysaccharide administration induces a rapid down-regulation of plasma
Reduced leukocyte CETP expression in ACS. Furthermore, a negative correlation between plasma CETP activity and the acute phase marker C-reactive protein (CRP) was observed during human clinical sepsis. Based on the high level of CETP production by macrophages and other BM-derived cells, leukocyte CETP may thus be involved in the acute phase response. Still, prospective data on CETP expression in circulating leukocytes in acute coronary syndromes (ACS) are lacking. Therefore, in the present study, we aimed to assess leukocyte CETP production in ACS. We show a strong correlation between diminished leukocyte CETP expression and episodes of unstable angina pectoris (UAP) in humans. Furthermore, induction of acute myocardial infarction (AMI) by coronary artery ligation in CETP Tg mice also resulted in a rapid down-regulation of leukocyte CETP mRNA expression. Together, these findings suggest a potential association between reduced leukocyte CETP expression and ACS.

MATERIALS AND METHODS

Tissue Harvesting and Immunohistochemical Analysis

Human tissues were fixed in paraformaldehyde, embedded in paraffin, and stored at room temperature at the department of Pathology, Leiden University Medical Center. The subsequent research was conducted in a coded and anonymous fashion, following the ethical guidelines as detailed by the FEDERA (National Federation of Scientific Council). Human tissues were cut into 8µm sections, which were subsequently stained with a primary CETP antibody TP-2 (a kind gift from Dr. Yves Marcel, Ottawa, Canada) and a peroxidase-conjugated secondary antibody (Jackson ImmunoResearch Laboratories, Suffolk, UK) or a secondary antibody conjugated to FITC (Jackson ImmunoResearch Laboratories, Suffolk, UK) for detection of CETP protein expression. Macrophages were visualized with a primary CD68 Ab-3 antibody (Thermo Fisher Scientific, Fremont, USA) and a horse radish peroxidase-conjugated secondary antibody (Emergo Europe, the Hague, The Netherlands).

Mouse tissues were collected from total-body CETP transgenic mice (CETP Tg; strain 5203; C57Bl/6J N10), expressing human CETP, controlled by its own promoter and natural flanking regions. Mice were fed regular chow diet, containing 4.3% (w/w) fat and no added cholesterol (RM3, Special Diet Services, Witham, UK). CETP Tg mouse tissues were stored in 3.7% neutral-buffered formalin (Formal-fixx; Shandon Scientific Ltd., UK) and 8µm cryostat sections were prepared. For detection of CETP protein expression cryostat sections were stained with a primary TP-2 and a secondary antibody conjugated to FITC (Jackson ImmunoResearch Laboratories, Suffolk, UK). Nuclei were stained with 4, 6-diamidino-2-phenylindole (DAPI) (Serva Feinbiochemica, Heidelberg, Germany). For double immunostaining, a primary monoclonal antibody F4/80 (BMA Biomedicals, Basel, Switzerland) and a Cy3-conjugated secondary antibody (Jackson ImmunoResearch Laboratories, Suffolk, UK) were used as macrophage marker. Photomicrographs were taken using a Bio-Rad Radiance 2100 MP confocal laser scanning system equipped with a Nikon Eclipse TE2000-U inverted fluorescence microscope. Non-fluorescent images were obtained with a
Leica image analysis system, consisting of a Leica DMRE microscope coupled to a camera and Leica Qwin Imaging software (Leica Ltd., Cambridge, UK).

**Study Population**
The APRAIS (Acute Phase Reaction and Ischemic Syndromes) study is a prospective study in patients displaying unstable angina pectoris (UAP). For the current study, 37 patients derived from this well-defined APRAIS study, who were admitted to the emergency department of Leiden University Medical Center between March and September 1995 with UAP Braunwald class IIIB were included and followed for up to 18 months. Additionally, 14 healthy age and sex matched volunteers were recruited. Venous blood samples were obtained on admission \((t=0)\) and at 180 days after admission \((t=180)\), centrifuged and plasma aliquots were stored at \(-80\)°C. All plasma samples were thawed only one single time prior to analysis. Furthermore, peripheral blood mononuclear cells (PBMCs) were isolated at different time points \((t=0\) and \(t=180)\) from venous EDTA blood samples through density centrifugation on Histopaque (Sigma, St. Louis, MO). Cells were cryopreserved in culture medium containing 20% FCS and 10% dimethylsulfoxide until further use. Explicit care was taken to prevent the use of previously deep frozen PBMC samples that had been exposed to defrosting. All patients received standard medical therapy, i.e. aspirin 300 mg orally, nitro-glycerine intravenously and heparin infusion titrated to the activated partial thromboplastin time. A clinical end point was the occurrence of refractory unstable angina during hospitalisation. UAP was considered refractory if angina in a resting condition, despite medical treatment, remained or re-occurred, prompting invasive coronary assessment and subsequent revascularization therapy. All subjects gave written informed consent and the study protocol was approved by the Ethics Committee of the Leiden University Medical Center.

**Inflamatory Marker Assays**
Data on the inflammatory markers, such as C-reactive protein (CRP), erythrocyte sedimentation rate and fibrinogen were obtained from the APRAIS database. Baseline CRP was measured in serum by a nephelometric method, range 0.2 to 1100 mg/l (N Latex CRP mono, Behring Diagnostics) as previously described, while CRP levels at \(t=180\) were measured via a turbidimetric assay on a fully automated Modular P800 U (Roche, Almere, The Netherlands). In addition, erythrocyte sedimentation rate and fibrinogen were determined as described in detail previously.

Serum amyloid A (SAA), another inflammatory marker was measured in CETP Tg mice by using a mouse SAA ELISA kit (Invitrogen Corporation, Carlsbad, California), according to manufacturer’s protocol.

**Murine Acute Myocardial Infarction**
CETP Tg mice (female, \(n=4\); male, \(n=4\), 20-25g) were anaesthetized and artificially ventilated (rate 200 breaths/min, stroke volume of 200ml) with a mixture of \(O_2\) and \(N_2O\) \([1:2\) (vol/vol)] using a rodent ventilator (Harvard Apparatus) containing 2–2.5% isoflurane for anesthesia. After a left anterior thoracotomy, myocardial infarction was induced by permanent ligation of the left anterior descending coronary artery with a sterile 7.0 silk suture (Prolene,
Johnson and Johnson, New Brunswick, NJ, USA). Age matched sham-operated mice (female, n=4; male, n=4) were utilized as controls. Sixteen hours after surgery, animals were sacrificed. PBMCs were isolated from 2ml EDTA blood samples, using lympholite-M (Cedarlane Laboratories Limited, Ontario, Canada), as recommended by the manufacturer. Serum was harvested for further analysis. For histological analyses, arterial trees of the ligated and sham-operated CETP Tg mice were perfused in situ with PBS. 8µm cryostat sections of the infarcted heart were routinely stained with hematoxylin (Sigma Diagnostics) & eosin (Merck Diagnostica, Darmstadt, Germany), or Masson’s trichrome (Sigma Diagnostics) staining. Neutrophil staining was performed using a naphthol AS-D chloroacetate esterase activity kit (Sigma-Aldrich, Zwijndrecht, The Netherlands) according to manufacturer’s instructions. Images were obtained with the Leica image analysis system as mentioned above. Animal experiments were performed at the Gorlaeus Laboratories of the Leiden/Amsterdam Center for Drug Research in accordance with the National Laws. All animal experimental protocols were approved by the Ethics Committee for Animal Experiments of Leiden University, and conformed to the principles of Laboratory Animal Care formulated by the Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85-23, revised 1996).

CETP Analyses
To assess leukocyte CETP mRNA and protein expression, quantitative RT-PCR and western blotting were performed on human and mouse PBMCs which were stored at -80°C until analysis in July 2007. In brief, guanidium thiocyanate-phenol was used to extract total RNA from PBMCs. Samples were subjected to DNase I treatment (Promega, Madison, WS) after which cDNA was generated using RevertAid M-MuLV reverse transcriptase (Fermentas, Burlington, Canada) according to manufacturer’s protocol. Quantitative gene expression analysis was performed using the SYBR-Green method on a 7500 fast Real-time PCR machine (Applied Biosystems, Foster City, CA). PCR primers (Table 1) were designed using Primer Express Software according to the manufacturer’s default settings. Cyclophilin and Hypoxanthine Guanine Phosphoribosyl Transferase (HPRT) were used as the standard housekeeping genes in the human studies and HPRT, β-actin, and acidic ribosomal phosphoprotein PO (36B4) in the murine studies. Relative gene expression was calculated by subtracting the threshold cycle number (Ct) of the target gene from the average Ct of housekeeping genes and raising two to the power of this difference, in order to exclude the possibility that changes in the relative expression were caused by variations in the separate housekeeping gene expressions. All used human and murine primer sequences are shown in Table 1a and 1b, respectively.

For Western blotting equal amounts of total PBMCs protein (20 µg) were run on a 10% SDS-PAGE gel and after electrophoretical transfer to Protran nitrocellulose membrane (Schleicher&Schnell, Dassel, Germany) CETP was detected using TP-2 as primary antibody and a secondary antibody conjugated to horseradish peroxidase (Jackson ImmunoResearch). Beta-tubulin antibody (Santa Cruz Biotechnology) was used as a loading control. Finally, immunolabeling was detected by enhanced chemiluminescence (Amersham Biosciences).
Furthermore, CETP mass was analyzed recently using a double-antibody sandwich enzyme-linked immunosorbent assay as described in detail previously.\textsuperscript{22}

**Table 1a. Human primers for quantitative real-time PCR analysis**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer</th>
<th>Reverse Primer</th>
</tr>
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<tbody>
<tr>
<td>HPRT</td>
<td>GAATGTCAGTGGCTGCTATTCTT</td>
<td>ACAATCCGCCCAAAGGGGAC</td>
</tr>
<tr>
<td>Cyclophilin</td>
<td>AGCTTGGCAAGTGCAAGTTGA</td>
<td>GAAGATGAGAAGCTACCTCAAGCTTCAAGCATCA</td>
</tr>
<tr>
<td>CETP</td>
<td>CAGATCAGCCACTTGTGGCA</td>
<td>CAGATCAGCCACTTGTGGCA</td>
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</table>

**Table 1b. Murine primers for quantitative real-time PCR analysis**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer</th>
<th>Reverse Primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-actin</td>
<td>AACCGTGAAAAGATGACCCAGAT</td>
<td>CACAGGCCTGGATGGCTACGTA</td>
</tr>
<tr>
<td>36B4</td>
<td>GACCCCGGAAAGACTGCTTTCCTT</td>
<td>GCACATCAGTGAATTTTCATGGA</td>
</tr>
<tr>
<td>HPRT</td>
<td>TGTGGTCAGTGGCTGCTGGA</td>
<td>AGGAGATGAGAAGCTACCTCAAGCATCA</td>
</tr>
<tr>
<td>CD68</td>
<td>CTTCCACCTTGCCTAGTC</td>
<td>TTGGGTATAAGTTCTGCAGAGTTGGA</td>
</tr>
<tr>
<td>CXCR4</td>
<td>GTGTATCTGTGCATGGTTT</td>
<td>TGACAGGTGCAGCCGCTTGA</td>
</tr>
<tr>
<td>CCR2</td>
<td>CCTTGGGAATGAGTAACTGCTGTGA</td>
<td>TGAGGAGATACCTGCAACTTCTCT</td>
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**Lipid Analysis**

The concentrations of free cholesterol (FC) and total cholesterol (TC) in plasma were determined recently using enzymatic colorimetric assays (Roche Diagnostics, Mannheim, Germany), with 0.025 U/ml cholesterol oxidase (Sigma) and 0.065 U/ml peroxidase and 15 µg/ml cholesteryl esterase (Roche Diagnostics, Mannheim, Germany) in reaction buffer (1.0 KPi buffer, pH=7.7 containing 0.01 M phenol, 1 mM 4-amino-antipyrine, 1% polyoxyethylene-9-lauryl ether, and 7.5% methanol). Precipath I was used as an internal standard. Absorbance was read at 490 nm. The distribution of cholesterol over the different lipoproteins in plasma was determined by fractionation of 30 ml plasma of each sample using a Superose 6 column (3.2x300mm, Smart-system, Pharmacia, Uppsala, Sweden). Cholesterol content of the effluent was determined as indicated.

**Statistics**

Baseline characteristics between stabilized and refractory UAP patients on admission were examined by the unpaired Student's t-test, and values represent means or means±SD. Plasma levels of inflammatory markers were tested for normal Gaussian distribution and values were log-transformed in case of a skewed distribution when appropriate. Regarding the latter, geometric instead of arithmetic means are given. Means were compared by unpaired two-tailed Student's t-test or Mann-Whitney U-test when appropriate. CETP mRNA expression in PBMCs of UAP patients (both stabilized and refractory) at t=0 and t=180 was compared by paired two-tailed Student's t-test, and values represent means±SEM. As for the murine AMI model, all data represent means±SEM. One way ANOVA and the Student Newman Keuls posttest were used to compare means after confirming normal Gaussian distribution. All analyses were performed using SPSS software (SPSS, Chicago, IL), and Graphpad Instat Software (San Diego, USA). A p value of <0.05 was considered significant.
RESULTS

Immunohistochemical localization of CETP in human and CETP Tg mouse tissues

In humans, CETP has been detected in various tissues. The liver is thought to be the main site of CETP synthesis in several species (e.g. humans, monkeys, rabbits), as well as in transgenic mice expressing human CETP under control of its own promoter and natural flanking regions. By immunohistochemistry, we revealed an intensive localization of CETP in hepatocytes of human livers (Fig. 1A). Interestingly, a striking zoned distribution of CETP expression was found with intense positivity around the central vein and periportal zone of the liver parenchyma. Furthermore, CETP protein expression was clearly detected in Kupffer cells of human livers, although it was not expressed by all Kupffer cells (Fig. 1A). In contrast, predominant Kupffer cell localization of CETP was observed in livers of total-body CETP Tg mice (Fig. 1C). In addition to the liver, CETP was visualized in other tissues (e.g. adipose tissue and spleen) of both humans and CETP Tg mice by immunohistochemical staining (Fig. 1B, C). Adipocytes and macrophages in the adipose tissue expressed CETP protein in humans as well as CETP Tg mice. Additionally, in line with our previous data in CETP Tg mice, CETP was detected in macrophages of the red pulp and in the B-cell rich marginal zone surrounding the white pulp of human spleens. Thus, both in humans and CETP Tg mice, CETP is expressed in tissue macrophages, which develop from circulating leukocytes.

Fig. 1A
Patient characteristics
Baseline patient characteristics were determined in plasma samples of 37 UAP patients (stabilized 21 vs. refractory 16) (Table 2). Upon admission (t=0), the stabilized and refractory UAP groups did not differ in age, risk factors, and history, except for a significantly lower body mass index in the refractory group vs. the stabilized group (24.2±2.4 vs. 26.8±4.2 kg/m²; p=0.04). The refractory UAP group, however, displayed a higher
Reduced leukocyte CETP expression in ACS

inflammatory status than the stabilized group, as illustrated by markedly elevated erythrocyte sedimentation rate (22.1 vs. 12.8 mm/h; \( p=0.02 \)), fibrinogen levels (3.83 vs. 3.19 g/l; \( p=0.01 \)), and moderately increased CRP levels (2.25 vs. 1.93 mg/l; \( p=0.71 \)). At 180 days after admission (\( t=180 \)), the erythrocyte sedimentation rate and fibrinogen levels were normalized to undetectable levels, and CRP levels were markedly decreased in both stabilized and refractory UAP patients (0.48 mg/l; \( p=0.02 \) and 0.95 mg/l; \( p=0.14 \), respectively), indicative of a transient inflammatory response and restoration of this response upon treatment of cardiac ischemia. These findings are in line with our previous report, showing a transient raise of the CC chemokine ligand-5 and -18 during cardiac ischemia.21

| Table 2. CETP cohort baseline patient characteristics and laboratory parameters |
|-------------------------------|-----------------|-----------------|--------------|
|                              | Total (N=37)    | Stabilized (N=21) | Refractory (N=16) |
| Male (%)                      | 68              | 57              | 81            |
| Age (years)*                  | 67.3 ± 11.1     | 65.6 ± 12.8     | 69.5 ± 8.3    |
| Body mass index (kg/m^2)*     | 25.6 ± 3.3      | 26.8 ± 4.2      | 24.2 ± 2.4    |
| Risk factors                  |                 |                 |               |
| Current smoker (%)            | 19              | 24              | 13            |
| Diabetes (%)                  | 11              | 10              | 13            |
| Hypertension (%)              | 32              | 38              | 25            |
| History                       |                 |                 |               |
| Myocardial infarction (%)     | 49              | 48              | 50            |
| PTCA (%)                      | 30              | 24              | 38            |
| CABG (%)                      | 32              | 29              | 38            |
| Laboratory parameters         |                 |                 |               |
| Hemoglobin (mmol/l)*          | 8.7 ± 0.8       | 8.8 ± 0.8       | 8.6 ± 0.8    |
| Hematocrite (%)*              | 0.42 ± 0.03     | 0.42 ± 0.03     | 0.41 ± 0.03  |
| Leukocytes (10^9/l)*          | 6.9 ± 1.9       | 6.6 ± 1.6       | 7.5 ± 2.3    |
| Platelet count (10^6/l)*      | 202.7 ± 54      | 192.9 ± 34      | 214.7 ± 37   |
| Glucose (mmol/l)*             | 6.6 ± 1.9       | 6.8 ± 2.2       | 6.4 ± 1.6    |
| Creatinine (µmol/l)*          | 110.1 ± 52      | 108.1 ± 61.9    | 112.6 ± 37.3 |
| Erythrocyte sedimentation rate (mm/h)**    | 16.2          | 12.8           | 22.1         |
| Fibrinogen (g/l)**            | 3.45            | 3.19           | 3.83         |
| C-reactive protein (mg/l)**   | 2.06            | 1.93           | 2.25         |

\*Values represent mean ± SD.
**Values denote geometric means;
\#P-value denotes statistical difference between stabilized and refractory patients upon admission.

The lipid profile in the refractory group vs. the stabilized group on admission demonstrated higher levels of TC (257±17 vs. 195±26 mg/dl; \( p<0.01 \)), FC (69±3 vs. 59±4 mg/dl; \( p=0.07 \)) and CE (188±9 vs. 137±13 mg/dl; \( p<0.01 \)), which was primarily caused by an increase in LDL cholesterol (LDL-C) (165±8 vs. 136±7 mg/dl; \( p=0.03 \). HDL-C was slightly but not significantly lower in refractory vs. stabilized patients (35±3 vs. 42±4 mg/dl; \( p=0.28 \)) (Table 3). Plasma lipid profiles of the UAP groups (both stabilized and refractory) were not significantly changed at \( t=180 \) vs. \( t=0 \). Furthermore, at \( t=180 \) no differences in lipid parameters were observed between stabilized and refractory patients. Healthy sex/age matched controls displayed markedly lower non-HDL cholesterol levels as compared to the UAP patients (both stabilized and refractory) at \( t=0 \) as well as at \( t=180 \). No differences in
HDL-C levels were observed between UAP patients and healthy control subjects (Table 3).

Table 3. Plasma lipid levels in a patient cohort displaying UAP on admission and 180 days post inclusion

<table>
<thead>
<tr>
<th>Population</th>
<th>Time (day)</th>
<th>N</th>
<th>TC (mg/dl)</th>
<th>FC (mg/dl)</th>
<th>CE (mg/dl)</th>
<th>HDL-C (mg/dl)</th>
<th>Non-HDL-C (mg/dl)</th>
<th>VLDL</th>
<th>LDL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stabilized UAP</td>
<td>0</td>
<td>21</td>
<td>195±26</td>
<td>59±4</td>
<td>137±13</td>
<td>42±4</td>
<td>30±3</td>
<td>136±7</td>
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<tr>
<td>Refractory UAP</td>
<td>180</td>
<td>21</td>
<td>229±33</td>
<td>67±5</td>
<td>162±16</td>
<td>49±5</td>
<td>32±3</td>
<td>156±11</td>
<td></td>
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<tr>
<td>Healthy Control</td>
<td>0</td>
<td>16</td>
<td>257±17**</td>
<td>69±3</td>
<td>188±9**</td>
<td>35±3</td>
<td>32±5</td>
<td>16±6</td>
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<tr>
<td></td>
<td>180</td>
<td>16</td>
<td>263±42</td>
<td>73±8</td>
<td>190±23</td>
<td>43±5</td>
<td>40±7</td>
<td>17±21</td>
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</table>

The amount of total, free and esterified cholesterol (TC, FC and CE), HDL cholesterol, and non-HDL cholesterol (VLDL+LDL) in UAP patients on admission (t=0) and 180 days post inclusion (t=180) as well as healthy sex/age matched controls is shown. Data represent mean±SEM. *p<0.05, **p<0.01 vs. stabilized patients at t=0. ξ p<0.05 vs. stabilized patients at t=0. †p<0.05, ††p<0.001 vs. refractory patients at t=0.

Reduced CETP expression in PBMCs from patients with UAP

Next, quantitative RT-PCR analysis was performed on mRNA from PBMCs isolated from the UAP patients at the time of admission (t=0) and at 180 days follow up (t=180) as well as from healthy sex/age matched controls (Fig. 2A). As compared to controls (0.006±0.002; n=14), patients from the stabilized and the refractory UAP group at t=0 displayed ~3-fold (0.002±0.001; p<0.01; n=21) and ~6-fold (0.001±0.0003; p<0.01; n=16) lower relative CETP mRNA levels in PBMCs, respectively (Fig. 2A). The refractory UAP group with the highest inflammatory status upon admission thus showed the lowest CETP mRNA expression in PBMCs. Western-blot analysis of PBMCs showed no CETP protein in PBMCs from both the stabilized and refractory UAP groups at t=0 (Fig. 2B). Importantly, CETP mRNA was significantly elevated in PBMCs of UAP patients at t=180 as compared to t=0 (0.019±0.006 vs. 0.002±0.001 and 0.019±0.005 vs. 0.001±0.0003 for the stabilized and refractory groups, respectively; p<0.01 in both cases). Beta-tubulin staining of Western blots showed a moderate increase in the amount of protein in PBMCs of UAP patients (both stabilized and refractory) at t=180 vs. t=0. However, dramatically elevated CETP protein levels could be observed as a clear single band (~70kDa) in all analyzed PBMC samples at t=180. PBMCs of stabilized and refractory subjects showed equally high CETP mRNA (Fig. 2A) and protein (Fig. 2B) expression levels at t=180. Normalization of the inflammatory status of UAP patients after standard medical therapy applied after hospital admission is thus associated with restoration of leukocyte CETP expression in both UAP groups and is independent of the re-occurrence of UAP in the refractory group.

Based on our previous observation in mice that CETP production by bone marrow-derived cells influences CETP mass in the circulation, we have analyzed plasma CETP mass levels. No marked difference was found among the stabilized and refractory UAP groups (both t=0 and t=180) and healthy controls (Fig. 2C).
Reduced leukocyte CETP expression in ACS

Fig. 2A

Fig. 2B

Fig. 2C

Fig. 2: Unchanged plasma CETP mass, but a markedly down-regulated expression of CETP in PBMCs of UAP patients. A: CETP mRNA expression in PBMCs of UAP patients at \( t=0 \) and \( t=180 \), compared with healthy sex/age matched controls, determined by RT-PCR. Values are the means±SEM. **\( p<0.01 \) vs. \( t=0 \), and ***\( p<0.01 \) vs. healthy controls. B: Western blotting for CETP protein in PBMCs. A representative immunoblot of 4 samples (stabilized=S, \( n=2 \); refractory=R, \( n=2 \)) at two time points is shown. A single band of CETP proteins (~70kDa) was observed in all tested PBMC samples at \( t=180 \), whereas this band was not observed in all corresponding samples at \( t=0 \). Beta-tubulin was used to control equal loading. C: Unchanged plasma CETP mass among UAP patients (at \( t=0 \) and \( t=180 \)) and healthy subjects. N.S. = non-significant.

Murine Acute Myocardial Infarction (AMI) Model

To further elucidate the mechanism of the observed reduced leukocyte CETP production in UAP patients at baseline, AMI was induced in CETP Tg mice by performing a permanent ligation of the left anterior descending coronary artery (LAD). First, we determined CETP mRNA expression in circulating leukocytes. In agreement with the reduced CETP expression observed in PBMCs of UAP patients upon admission to the hospital, a dramatic reduction in leukocyte CETP mRNA expression was found in the AMI vs. the sham-operated group (0.04±0.01 vs. 0.09±0.02; \( p<0.05 \)). Interestingly, also the sham-operated group showed diminished CETP expression as compared to controls without any operation (0.09±0.02 vs. 0.19±0.02; \( p<0.05 \) (Fig. 3A).
In humans, CRP is regarded as the prototype of acute-phase proteins. However, CRP is expressed only at a very low concentration in mice and does not show an acute phase behavior. Therefore, instead of CRP, another acute phase marker serum amyloid A (SAA) was measured in control, sham-treated, and AMI-induced mice (Fig. 3B). SAA was markedly increased in sham-treated mice vs. controls (9.24±0.54 vs. 0.04±0.03 g/l; p<0.0001). In addition, AMI induced a further increase in SAA levels as compared to the sham-operated group (12.28±0.27 vs. 9.24±0.54 g/l; p<0.01). Interestingly, the inflammation marker SAA showed a strong negative correlation with leukocyte CETP mRNA expression (r=0.87, p=0.0025), thereby further supporting the hypothesis that the observed reduction in leukocyte CETP expression is correlated with enhanced inflammation.

Leukocytes, including macrophages, monocytes and neutrophils play an important protective role in myocardial wound healing. In agreement, at 16 hours after LAD ligation, extensive accumulation of neutrophils was observed in the infarcted myocardium as compared to the myocardium of sham-operated mice (Fig. 4A). In line with this finding, a ~5-fold (p<0.05) higher CXCR4 mRNA expression was observed (Fig. 4B). In addition, CD68 and CCR2 mRNA expression in the infarcted zone were enhanced ~44-fold (p<0.05) and ~20-fold (p<0.001), respectively (Fig. 4B). Due to the enhanced leukocyte recruitment as indicated by the observed dramatic increase in CXCR4, CD68, and CCR2 mRNA, ~20-fold (p<0.05) increased CETP mRNA expression was observed in the infarcted heart (Fig. 5A). Calculation of the CETP/CD68 ratio, indicative for macrophage CETP expression, showed a 53% reduction in the myocardium of the AMI vs. the sham-operated group (0.92 vs. 1.97) (data not shown). Furthermore, hepatic CETP mRNA was ~2-fold (p<0.01) lower in the AMI group, and a tendency to reduced CETP mRNA expression was observed in the adipose tissue and spleen of the AMI group, but these failed to reach statistical significances (Fig. 5B).
Fig. 4: Characterization of AMI model. A: Immunostaining showed marked elevation in the number of neutrophils (red arrows) in the myocardium of mice subjected to coronary artery ligation for 16 hours, which could not be detected in the myocardium of sham-operated mice. Original magnification ×400. B: Significant elevated mRNA expression of CXCR4, CD68, and CCR2 was found in the infarcted heart, determined by RT-PCR. Values are the mean±SEM of 8 mice per group. *p<0.05, ***p<0.001 vs. sham-operated mice.

Fig. 5: Effect on CETP expression after the induction of AMI in CETP Tg mice. A: Significantly enhanced CETP mRNA in the infarcted heart after AMI. B: Impaired CETP mRNA expression in the liver, adipose tissues and spleen after AMI, determined by RT-PCR. Values are the mean±SEM of 8 mice per group. *p<0.05, **p<0.01 vs. sham-operated mice.
In agreement with the human studies, no significant change was found in circulating CETP mass between AMI and sham-operated mice (7.35±0.09 vs. 6.99±0.21 µg/ml; p=0.69). Furthermore, no significant changes in the levels of TC (79±8 vs. 71±5 mg/dl) and CE (48±7 vs. 51±5 mg/dl) were observed. FC levels were moderately but not significantly increased (32±6 vs. 20±3 mg/dl; p=0.09) in the AMI vs. the sham-operated group. HDL-C levels did not differ significantly between the AMI and sham-operated group (49±6 vs. 48±11 mg/dl) (Table 4).

Table 4. Plasma lipid levels after the induction of acute myocardial infarction (AMI) in CETP Tg mice

<table>
<thead>
<tr>
<th>Group</th>
<th>Time (hour)</th>
<th>N</th>
<th>TC (mg/dl)</th>
<th>FC (mg/dl)</th>
<th>CE (mg/dl)</th>
<th>HDL-C (mg/dl)</th>
<th>Non-HDL-C (mg/dl)</th>
<th>VLDL</th>
<th>LDL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham</td>
<td>16</td>
<td>8</td>
<td>71±5</td>
<td>20±3</td>
<td>51±5</td>
<td>48±11</td>
<td>5±1</td>
<td>17±2</td>
<td></td>
</tr>
<tr>
<td>AMI</td>
<td>16</td>
<td>8</td>
<td>79±8</td>
<td>32±6</td>
<td>48±7</td>
<td>49±6</td>
<td>6±2</td>
<td>19±3</td>
<td></td>
</tr>
</tbody>
</table>

Blood samples were drawn without fasting at 16 hours post-ligation while feeding regular chow diet. Sera from individual mice were measured for HDL cholesterol levels. The amount of total, free and esterified cholesterol (TC, FC and CE), HDL cholesterol as well as non-HDL cholesterol (VLDL + LDL) in CETP Tg mice is shown. Data represent mean±SEM of 8 mice per group. No significant difference was observed between the AMI and sham-operated groups.

DISCUSSION

To our knowledge, this is the first study to describe a potential association between leukocyte CETP and acute coronary syndromes (ACS) in a prospective manner. Until now, only a few studies have evaluated the function of CETP in ACS. For instance, decreased plasma CETP levels were demonstrated in humans during the acute phase response after cardiac surgery, while significantly higher CETP concentrations were described in survivors of a myocardial infarction or stroke. Of note, a protective effect of the B2B2 genotype of the CETP Taq1B polymorphism on the likelihood of having a first event of ACS in normal-weight persons has been reported recently. To date, however, little is known about the relationship between CETP and ACS. In the present study, UAP patients displayed significantly diminished levels of CETP expression in PBMCs upon admission as compared to healthy subjects. The direct cause for the reduced CETP expression after onset of ischemia is currently unknown. It may be speculated that the increased inflammatory response observed in the UAP patients as evidenced by elevated levels of several inflammatory parameters (e.g. erythrocyte sedimentation rate, fibrinogen, and CRP) may relate to the observed decrease in CETP expression. Most importantly, both in the stabilized and refractory UAP group, CETP production in PBMCs was markedly increased after the half-year period with medical therapy leading to normalization of the inflammatory status. Thus, the reduced leukocyte CETP expression in UAP patients as compared to healthy subjects, as well as the restoration of leukocyte CETP expression and decreased inflammatory status in exactly the same population of UAP patients at 6 months after initiation of treatment of the UAP symptoms (t=180 vs. t=0) suggest that leukocyte CETP expression might be an indicator of the inflammatory response associated with UAP. Endotoxin administration, which mimics gram-negative bacterial infection, also induces a rapid decrease in CETP levels both in mice expressing the human CETP gene and in hamsters with
Reduced leukocyte CETP expression in ACS

Furthermore, a negative correlation between plasma CETP activity and CRP was observed during human clinical sepsis. Reduced leukocyte CETP expression might thus be common to different inflammatory stimuli and it can be hypothesized that leukocyte CETP might serve an important function in the inflammatory/immune response. Importantly, also in our murine AMI model, sham-treated mice showed significantly decreased leukocyte CETP mRNA expression and dramatically increased SAA levels as compared to controls without any operation. AMI-induced mice with the lowest leukocyte CETP expression showed the highest SAA levels, which corresponds with the observed markedly lower CETP production in PBMCs of UAP patients upon admission. Increased SAA, like CRP, has also been shown to reflect systemic inflammation. The strong negative correlation between leukocyte CETP expression and the inflammation marker SAA in our murine AMI model confirms our hypothesis that impaired leukocyte CETP production in ACS occurs both in humans and in CETP Tg mice, and it is associated with an enhanced inflammatory status.

Although leukocyte CETP expression was markedly reduced in UAP patients, plasma HDL-C and circulating CETP mass were unchanged. Previously, we have shown in CETP Tg mice that Kupffer cells which contribute to only 2.5% of the total liver protein, contain at least 48% of the total liver CETP expression, as compared with 38% and 14% for parenchymal cells and endothelial cells, respectively. In contrast to our findings in mice, in the current study, we revealed an intensive localization of CETP in hepatocytes of human livers in addition to expression in Kupffer cells. Thus, although in mice expressing human CETP under control of its natural regulatory elements, macrophages and other bone marrow-derived cells are important contributors to the circulating CETP mass, this may be less relevant in humans. However, also no effect of reduced leukocyte CETP expression was observed on circulating CETP mass in our murine AMI model. Suppression of CETP expression in circulating leukocytes and several tissues (especially the liver) paralleled an enhanced expression of CETP in the infarcted heart as a result of a dramatically enhanced recruitment of leukocytes after AMI. Thus, after AMI the balance in CETP production by the different organs may have shifted, ultimately leading to similar amounts of CETP protein secreted into the circulation.

As HDL has a protective function in atherosclerosis and cardiovascular disease, pharmacological inhibition of CETP is being studied as a method to induce HDL levels. Torcetrapib, a potent CETP inhibitor, increases HDL levels and inhibits the development of atherosclerosis in rabbits. In early-phase studies in humans, the drug also increased HDL-C (≥60%) and moderately lowered LDL-C. Strikingly, the publication of the ILLUMINATE trial (Investigation of Lipid Level management to Understand its Impact In Atherosclerotic Events) recently indicated that Torcetrapib treatment on top of Atorvastatin increased the risk for death and cardiovascular disease events in high-risk patients. The lack of efficiency of Torcetrapib is suspected to be due to: 1) off-target effects of Torcetrapib, unrelated to CETP inhibition, including the abnormally high blood pressure and increased levels of serum sodium, bicarbonate, and aldosterone; and/or 2) an adverse effect of CETP inhibition per se, with the possible generation of dysfunctional or even proatherogenic HDL-C. Interestingly, significantly more
Torcetrapib/Atorvastatin than Atorvastatin-only treated patients died of infection (9 vs. 0), which implies that CETP inhibitors could affect the immune system. Since our data strongly suggest an association between leukocyte CETP expression and ACS-induced inflammation, the question arises whether reduced leukocyte CETP production is also causal in the negative effects of Torcetrapib treatment. With the continued development of other CETP inhibitors like JTT-705 and MK-0859 (Anacetrapib) as a potential new class of drugs for the treatment of cardiovascular risk, it is thus essential that the effects of CETP inhibition on leukocyte function are carefully addressed.

To conclude, in the current study, we propose that ACS-induced inflammation is one of the major causative factors for reduced leukocyte CETP expression. Understanding the mechanisms behind the impairment of leukocyte CETP production and the importance for the inflammatory/immune response and possibly the pathogenesis of ACS will not only provide insight into this issue but will also yield important information on the effects of therapeutic inhibition of CETP on leukocyte functions.

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**REFERENCE**

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