Human cholesteryl ester transfer protein measured by enzyme-linked immunosorbent assay with two monoclonal antibodies against rabbit cholesteryl ester transfer protein: plasma cholesteryl ester transfer protein and lipoproteins among Japanese hypercholesterolemic patients

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Plasma cholesteryl ester transfer protein (CETP) concentrations were measured in Japanese subjects by an ELISA with two different monoclonal antibodies that were raised against rabbit CETP and cross-reacted against human CETP. Among 63 patients who consecutively underwent coronary angiography, the plasma CETP of 37 patients with luminal stenosis ≥50% in their coronary arteries was not significantly different from that of the 26 patients with luminal stenosis <50%. No other lipoprotein-related measurement except HDL-cholesterol differentiated the two groups. Among 40 hypercholesterolemic patients, no lipoprotein-related measurement other than LDL-cholesterol was found to positive correlate with the CETP. Before and after the treatment of 23 patients with simvastatin 5 mg a day for 4 weeks, plasma CETP markedly decreased in those whose pretreatment CETP was ≥3 mg/L; no change was observed for those with lower pretreatment CETP. In the former group, negative correlation between CETP and HDL-cholesterol was demonstrated only in the posttreatment plasma.

Cholesteryl ester transfer protein (CETP) mediates the transfer/exchange of cholesteryl ester (CE) and triglyceride (TG) between plasma lipoproteins (1–3). Because CE is mainly generated by lecithin: cholesterol acyltransferase in HDL in plasma (4), the hetero-exchange of CE with TG by CETP leads to the net CE transfer from HDL to apolipoprotein B-containing lipoproteins. This reaction is believed to be one of the key steps of cholesterol transport from peripheral tissues to the liver, which is proposed to involve cellular cholesterol efflux to HDL, its esterification in HDL, CE transfer to other lipoproteins, and eventually, the uptake of the lipoproteins by the liver via receptor-mediated processes (5, 6). The pathway is of physiological importance because the cholesterol molecule is not catabolized in the peripheral tissues except for the steroidogenic cells, and thus CETP is expected to play an important role in cholesterol homeostasis.

In such a context, CETP would help the removal of cholesterol from an atherosclerotic vascular lesion. However, it also lowers HDL and may raise LDL in plasma by transferring CE from HDL, which is inconsistent with the expected beneficial function of CETP in counteratherogenesis. This issue is indeed still controversial in animal experiments and clinical observations. The reports on atherosclerosis in transgenic mice expressing CETP are in conflict (7, 8). A heterozygous genetic defect of CETP is reportedly atherogenic when compared on the same HDL concentration (9). A few more recent reports indicated that the CETP deficiency is not protective against atherosclerosis despite the marked hyperalphaproteinemia that accompanies it (10, 11). Thus, more clinical data are needed to understand the relationship of CETP.
to atherosclerosis. A reliable method to measure plasma CETP concentrations is therefore primarily important.

We have raised monoclonal antibodies (mAbs) against CETP isolated from rabbit plasma, and two of them cross-reacted with human CETP (12, 13). In this study, we report a new ELISA method that uses these two mAbs, 3–11D and 14–8F. We also include the results of CETP measurement using this method in plasma samples from patients with coronary heart disease and hypercholesterolemia.

Materials and Methods

Specific anti-CETP mAbs, mAb 3–11D and mAb 14–8F, have been raised against rabbit CETP and characterized (13). MAb 3–11D inhibited both [3H]CE and [3H]TG transfer from LDL to HDL by human CETP. This antibody seems to react against a conformational epitope of human CETP, because binding in immunoblots has been detected only in the absence of sodium dodecyl sulfate. MAb 14–8F inhibited only [3H]TG but not [3H]CE transfer by human CETP and reacted against a linear epitope of human CETP, which was indicated by the positive immunoblot binding in the presence of sodium dodecyl sulfate. Production of each mAb was amplified by intraperitoneal injection of the clonal hybridomas in RPMI-1640 (2 × 10^7 cells) into pristane-primed BALB/c mice (pristane purchased from Sigma Chemical Co.), and the ascites fluid was harvested 2 weeks later. The antibodies (IgG) were purified by protein G-Sepharose 4 (Pharmacia Biotech) chromatography.

CETP was isolated from human plasma by Dr. Taira Ohnishi, Kagawa Medical University, Miki-cho, Japan, according to the method previously described (14). The plasma of the homozygous patient with CETP deficiency (intron 14 splicing defect) (15) was kindly provided by Dr. T. Miida, Niigata University, School of Medicine, Niigata, Japan.

MAb 3–11D (0.5 μg in 50 μL of 50 mmol/L sodium bicarbonate, pH 9.6) was put into microwells of the assay plates (Falcon PRO-BIND™ Assay Plates, 96 flat-bottom wells, Becton Dickinson) and left at 4 °C overnight. The unbound antibody was removed, the wells were rinsed twice with phosphate-buffered saline (PBS; 20 mmol/L sodium phosphate buffer, pH 7.4, containing 154 mmol/L NaCl) to which 0.5 mL/L Tween 80 was added (PBS-T), and the blocking solution of 10 g/L bovine serum albumin and 14 g/L NaN₃ in PBS was added. The blocking mixture was left for 1 h at 37 °C and removed, and the wells were rinsed three times with PBS-T. The samples, appropriately diluted in 20 mmol/L phosphate buffer containing 1 mmol/L NaCl and 0.5 mL/L Tween 80, were then added at 50 μL/well and allowed to stand at 37 °C for 4 h. The antigen solution was removed, and the plates were rinsed five times with PBS-T. The detection antibody, mAb 14–8F (0.5 μg conjugated with horseradish peroxidase) (16), was added to 50 μL of the same sodium phosphate buffer as the samples. After incubation for 1.5 h at 37 °C, the unbound antibody was removed, and the wells were rinsed five times with PBS-T. The freshly prepared color reagent solution (0.4 g/L o-phenylenediamine and 0.6 g/L hydrogen peroxide solution in 58 mmol/L sodium phosphate, 21 mmol/L citrate buffer, pH 5.6) was added, and the plate was incubated for 20 min at 37 °C. The reaction was stopped by adding 50 μL of 2 mol/L sulfuric acid. The absorbance at 490–650 nm was measured for each well in a SPECTRE MAX 340™ (Molecular Devices).

For the coronary trial, 63 Japanese men between 31 and 59 years of age (51 ± 6 years, mean ± SD) consecutively underwent elective coronary angiography for diagnosis of anginal chest pain at the Nagoya City University Hospital. The coronary angiogram of each patient was reviewed by a panel of three cardiologists blinded to the identity of subjects, their clinical histories, and laboratory data. The subjects were classified into two groups: those who had luminal stenosis ≥50% in one or more coronary arteries (group 1) and those who had stenosis <50% (group 2). Blood samples were taken before angiographic examination. For the hyperlipidemia trial, 40 Japanese patients, 16 men and 24 women between the ages of 26 and 81 years (55 ± 14.4 years, mean ± SD), were treated for 8 weeks with dietary calorie intake restricted to 30 cal/kg standard weight (22 × [height (m)]²) with a composition of 26.6% protein, 20.3% fat, and 53.1% carbohydrate by weight. Additional treatment with 5 mg a day of simvastatin for 4 weeks was given to the 23 patients (4 men and 19 women) who were willing to take the drug among those resistant to dietary treatment and who had LDL-cholesterol >1.2 g/L. Blood samples were obtained at the end of the dietary treatment and at the end of the drug period. All the patients who participated in the trials provided written informed consent.

Fasting venous blood was collected into EDTA-containing glass tubes. Plasma total cholesterol and TG concentrations were determined by enzymatic methods. The plasma HDL-cholesterol concentration was measured after precipitation of apolipoprotein B-containing lipoproteins with dextran sulfate and magnesium chloride. Plasma LDL-cholesterol was calculated according to the equation of Friedewald et al. (17). CETP activity was measured by the method described by Albers et al. (18), monitoring the transfer of radiolabeled CE (14C-cholesteryl oleate, New England Nuclear) from the CE-donor HDL to the unlabeled acceptor LDL.

Results

No CETP was detected by this method in the plasma of a homozygote of genetic CETP defect because no increase of absorbance was observed over a 10- to 500-fold range of plasma dilutions. This plasma was used as a carrier of the CETP isolated from human plasma for the primary calibrator of the assay, which was prepared as 2, 4, and 8 mg/L. The plot of the absorbance at 490–650 nm against the logarithm of CETP concentration by dilution of the
each calibrator solutions gave an identical linear relationship (data not shown). When this primary calibration curve was used, the CETP concentration of normolipidemic human plasma was determined as 1.75 mg/L, using a 1:25 sample dilution. Dilution of this plasma yielded a linear calibration superimposable on that of the primary calibrators; therefore, this plasma was used as the secondary standard. On the basis of this secondary calibrator, the mean plasma CETP concentration of 20 normolipidemic subjects was $1.92 \pm 0.53$ mg/L (range $0.84 - 3.03$ mg/L; Table 1 and Fig. 1). Precision of the assay was estimated by analyzing a single plasma eight times in the same microwell plate and by running eight assays of the same plasma over a 10-day period. Intra- and interassay coefficients of variation were 3.5% and 6.2%, respectively.

Table 2 shows a summary of the subjects with coronary heart disease. Group 1 represents those who had luminal stenosis $\geq 50\%$ in one or more coronary arteries (37 men). Group 2 includes those who had stenosis $<50\%$ (26 men). The concentration of HDL-cholesterol was significantly higher in the Group 2 than the Group 1 ($P < 0.05$). Otherwise, there was no significant difference in total cholesterol, LDL-cholesterol, TG, and CETP activity between the two groups. CETP concentration showed neither a difference between the two groups nor a correlation with HDL-cholesterol in either group. CETP concentration of either group was not different from that separately measured for the reference subjects mentioned earlier. The distribution profiles of the CETP of groups 1 and 2 are shown in Fig. 2. There may be a slight shift of the peak to the higher concentration in group 2, which has not reached statistical significance.

Forty hypercholesterolemic patients were instructed to follow a restricted calorie intake for an 8-week period. Among those in whom diet therapy failed to decrease LDL-cholesterol to 1.2 g/L or less, 23 patients took simvastatin 5 mg a day. Table 3 summarizes the plasma lipids, lipoprotein, and CETP concentration, as well as the CETP activity, of these patients. The average CETP concentration of the original 40 patients was higher than that of the normolipidemic control group, although not significantly ($2.13 \pm 1.01$ vs $1.92 \pm 0.53$ mg/L). This may have been due to the subgroup of patients who had a high concentration of CETP (Fig. 3A). This view was supported by significant positive correlation between the CETP and LDL-cholesterol concentrations ($r = 0.555, P < 0.001$; Fig. 3B). However, there was no significant correlation between CETP and HDL-cholesterol among these patients (Fig. 3C). As mentioned above, 23 of the 40 patients also underwent the simvastatin treatment. Lipid and lipoprotein values and CETP were monitored after the 4-week treatment (Table 3). Before the drug treatment, the CETP concentration of this subgroup was higher than that of those who did not receive the drug therapy ($2.57 \pm 0.77$ vs $1.53 \pm 1.02$ mg/L, $P < 0.001$), perhaps reflecting their plasma LDL-cholesterol concentration. By the end of the treatment with simvastatin, plasma total cholesterol, LDL-

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**Table 1. Clinical characteristics of the normolipidemic subjects (n = 20).**

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Value</th>
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<tbody>
<tr>
<td>Age, years</td>
<td>$32 \pm 8^a$</td>
</tr>
<tr>
<td>Total cholesterol, g/L</td>
<td>$1.87 \pm 0.30$</td>
</tr>
<tr>
<td>Triglycerides, g/L</td>
<td>$0.91 \pm 0.38$</td>
</tr>
<tr>
<td>HDL-cholesterol, g/L</td>
<td>$0.56 \pm 0.12$</td>
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<tr>
<td>LDL-cholesterol, g/L</td>
<td>$1.13 \pm 0.28$</td>
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<tr>
<td>CETP, mg/L$^b$</td>
<td>$1.92 \pm 0.53$</td>
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</table>

$^a$ Mean $\pm$ SD.

$^b$ Mass concentration in plasma.

**Table 2. Clinical characteristics of the 63 male patients who underwent elective coronary angiography.**

<table>
<thead>
<tr>
<th>Group 1 (n = 37)</th>
<th>Group 2 (n = 26)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, years</td>
<td>$52 \pm 5^b$</td>
</tr>
<tr>
<td>T-chol, g/L$^c$</td>
<td>$1.94 \pm 0.37$</td>
</tr>
<tr>
<td>TG, g/L</td>
<td>$1.68 \pm 1.04$</td>
</tr>
<tr>
<td>HDL-C, g/L</td>
<td>$0.42 \pm 0.12$</td>
</tr>
<tr>
<td>LDL-C, g/L</td>
<td>$1.19 \pm 0.31$</td>
</tr>
<tr>
<td>CETP, mg/L</td>
<td>$1.76 \pm 0.60$</td>
</tr>
<tr>
<td>CETA, %/100 μL/2 h</td>
<td>$20.9 \pm 6.1$</td>
</tr>
</tbody>
</table>

$^a$ Group 1 includes 37 patients who had luminal stenosis $\geq 50\%$ in one or more coronary arteries, and group 2 includes 26 patients who had stenosis $<50\%$.

$^b$ Mean $\pm$ SD.

$^c$ T-chol, total cholesterol; HDL-C, HDL-cholesterol; CETA, cholesteryl ester transfer activity, given as percent CE transferred per 100 μL of plasma per 2 h.

$^d$ Significantly different from the values of group 1, $P < 0.05$.  

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![Histogram of the plasma CETP concentrations of normolipidemic control subjects (see Table 1).](image)
cholesterol, and CETP activity decreased, whereas HDL-cholesterol increased significantly (Table 3).

The average CETP concentration was also decreased by the treatment (Table 3). The distribution profile of the CETP concentration indicated the two distinct subgroups of CETP concentration in the pretreatment stage (Fig. 4 and Table 4). As shown in Fig. 4A1, patients of group A whose pretreatment concentration was $<3$ mg/L showed essentially the same distribution profile as those of the normolipidemic control group ($2.10 \pm 0.44$ vs $1.92 \pm 0.53$ mg/L), whereas the eight patients (all female) in group B (Fig. 4B1) were distinct from group A, having a pretreatment concentration $\geq 3$ mg/L. The CETP concentration was not substantially changed by the treatment in group A ($2.01 \pm 0.52$ mg/L) despite the change of LDL and HDL (Fig. 4A2). In contrast, the CETP concentration of group B was markedly decreased by the simvastatin treatment, from $3.46 \pm 0.29$ mg/L (range, $3.06 - 3.77$ mg/L) to $2.46 \pm 0.64$ mg/L (range, $1.21 - 3.32$ mg/L; $P = 0.0074$ by $t$-test and 0.012 by Wilcoxon test; Fig. 4B2). No apparent difference was demonstrated between groups A and B in their lipid and lipoprotein profile before and after the treatment (Table 4). In group B, significant correlations between CETP and HDL-cholesterol appeared in the post-treatment stage ($r = 0.81$). No significant correlation was observed between CETP and HDL-cholesterol for the pretreatment condition in either group or between post-treatment CETP and HDL-cholesterol in group A.

**Discussion**

CETP is one of the major potential determinants of plasma lipoprotein profile by its action in transferring cholesteryl ester among lipoproteins (3). However, it is unclear how CETP is involved in physiological regulation of plasma lipoprotein profile and whether CETP is anti- or proatherogenic, as mentioned earlier.

To investigate this problem in clinical studies, various methods have been reported for immunomeasurement of the CETP mass in plasma (19–27). Not many methods reported have used polyclonal antibodies, perhaps because the CETP isolated from human plasma has not been completely proven homogeneous. Immunoassay of human CETP was first established as a competitive solid-phase radioimmunoassay by the use of a mAb against human plasma CETP (20), and it was used to demonstrate the lack of CETP in the plasma of those with its genetic defect (15). Another report introduced second oligoclonal antibodies raised against the fragment peptides of CETP for a sandwich-type immunoassay (26). Thus, availability of antibodies suitable for the immunoassay seemingly is

<table>
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<th>Table 3. Clinical characteristics of the hyperlipoproteinemic patients. a</th>
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<tr>
<td><strong>Dietary management</strong></td>
</tr>
<tr>
<td>Before (n = 40)</td>
</tr>
<tr>
<td>T-chol, g/L b</td>
</tr>
<tr>
<td>TG, g/L</td>
</tr>
<tr>
<td>HDL-C, g/L</td>
</tr>
<tr>
<td>LDL-C, g/L</td>
</tr>
<tr>
<td>CETP, mg/L</td>
</tr>
<tr>
<td>CETA, %/10 $\mu$L/3 h</td>
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</table>

a Forty subjects underwent the dietary management for 8 weeks to reach the baseline condition, and 23 of them received further drug treatment with simvastatin 5 mg a day for 4 weeks.

b T-chol, total cholesterol; HDL-C, HDL-cholesterol; CETA, cholesteryl ester transfer activity, given as % CE transfer by 10 $\mu$L of plasma per 3 h.

c Mean $\pm$ SD.

d Significantly different from the values before the drug treatment, $P < 0.0001$.

* Significantly different from the values before the drug treatment, $P < 0.05$.  

**Fig. 2.** Distribution of the plasma CETP concentrations of patients with coronary stenosis.  
Group 1 (left panel) included 37 patients who had the luminal stenosis $\geq 50\%$ in one or more coronary arteries, and group 2 (right panel) included 26 patients who had stenosis $<50\%$ (see Table 2). The curved lines indicate the gaussian curve fit calculated from the distribution profiles.
of primary importance, and the mAbs stably expressed by the established cell lines should be the most reliable source of the antibodies. An enzyme-linked colorimetric system would be an additional advantage, avoiding the use of radioisotopes to make the assay system conventional. Therefore, we have established a new ELISA for CETP in human plasma by using two different mAbs, 3–11D and 14–8F, both of which had been raised against rabbit plasma CETP and characterized for binding to human CETP and inhibition against the lipid transfer by human CETP (13).

Using this technique, we have measured the plasma CETP concentration of patients with coronary heart disease. The distribution profile of plasma CETP did not reach significant statistical difference between the patient groups with coronary stenosis of different degrees of severity, whereas HDL-cholesterol clearly differentiated these groups. Plasma CETP was also measured in hypercholesterolemic patients before and after simvastatin treatment. In agreement with previous observations (28, 29), CETP showed positive correlation with plasma LDL concentrations and no correlation with the HDL concentration. The hypercholesterolemic patients apparently included a subgroup, with high CETP concentrations, who were distinct from those having a CETP distribution within reference values. The simvastatin treatment markedly reduced the CETP concentration of the high CETP group but did not affect the concentration of the group whose pretreatment CETP concentration was within reference values. These results are consistent with the reports that simvastatin reduced the average plasma CETP activity in hyperlipoproteinemia but showed no clear correlation between the changes in CETP activity and plasma lipoproteins (30) and that cholestyramine treatment of hypercholesterolemic patients produced a reduction of their plasma CETP concentrations (31).

The reason for the positive correlation between plasma CETP activity and LDL-cholesterol (28, 29) is unknown, whether LDL increases as a result of transfer of CE from HDL or CETP is increased by the increase of LDL. Inconsistency has also been observed in some pathological states: decreased CETP activity in non-insulin-dependent diabetes mellitus patients (32) but increased activity in insulin-dependent diabetes mellitus patients (33), despite the high LDL in both groups; reduced CETP activity in chronic renal diseases (34); and the decrease in CETP mass in patients treated with hemodialysis (35), in spite of the increase of their LDL.

Changes in CETP concentration throughout those with the normal CETP genotype and heterozygotes of CETP deficiency are unlikely to contribute to direct regulation of HDL-cholesterol, although complete CETP deficiency results in a very high HDL-cholesterol concentration (36, 37). However, the plasma CETP concentration correlates with HDL-cholesterol in hypertriglyceridemic humans (38) and monkeys (39), indicating a notable contribution of the exchange of CE with TG to the regulation of HDL-cholesterol by CETP. Nevertheless, the slight increase of CETP by probucol has been viewed as a potential cause of the HDL reduction by this drug (40, 41).

Many other metabolic factors may influence the plasma CETP concentration independently. Chronic alcoholic intake reduces CETP activity, which may account for
the increase of HDL (42, 43). The effect of cigarette smoking is controversial (44, 45), as is the role of estrogen (20, 28, 46, 47). Reports are also controversial about correlation of CETP activity with the body mass index (48, 49).

The most important clinical questions is whether CETP plays a major role in atherogenesis. The finding with transgenic mice that expressed monkey CETP supported the hypothesis that CETP is atherogenic (7), whereas the finding with human CETP-transgenic mice did not (8). On the other hand, inhibition of CETP prevented atherogenesis in cholesterol-fed rabbits (27). However, the study on the heterozygotes of the genetic CETP deficiency showed that low CETP has negative impact on the prevention of coronary heart disease as long as the HDL-cholesterol concentration matches that of the control (0.4–0.6 g/L) (9). More recent studies by Hirano and co-workers (10, 11) also concluded that CETP deficiency was not necessarily antiatherogenic. No definitive data are available for the

Fig. 4. Distribution of the CETP concentrations of hypercholesterolemic patients in the predrug (upper two panels) and postdrug (lower two panels) stages of treatment with simvastatin 5 mg a day for a 4-week period.

(A1 and A2) Patients in group A with pretreatment CETP < 3 mg/L before (A1) and after (A2) the drug treatment. (B1 and B2) Patients in group B with pretreatment CETP ≥ 3 mg/L before (B1) and after (B2) the drug treatment (see Table 4).
role of CETP in atherogenesis among those with the unaffected CETP gene.

Thus, more clinical data are needed for understanding the role of CETP in atherogenesis. The ELISA described in this report would provide a new conventional tool for measuring CETP concentrations in various biological samples. In our preliminary observation, plasma CETP concentration is neither a regulating factor of HDL concentration nor an indicative risk for coronary heart disease. Also implicated is that there is a subgroup of hypercholesterolemic patients who have distinctly high plasma CETP concentrations. Treatment of hypercholesterolemia of such patients produces a notable reduction of plasma CETP concentrations. Treatment of hypercholesterolemia of such patients produces a notable reduction of CETP, whereas it has little influence on the CETP of the group with CETP concentrations within reference values. Thus, the CETP concentration should be analyzed more carefully in clinical trials, taking into account the heterogeneous background of the plasma CETP concentration.

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References


