# Quantitative and compositional changes in high density lipoprotein subclasses in patients with various genotypes of cholesteryl ester transfer protein deficiency

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**Abstract** High density lipoprotein (HDL) with and without apolipoprotein (apo) E was quantified and characterized in subjects with three genotypes of cholesteryl ester transfer protein (CETP) deficiency: the nonsense mutation in intron 14 (10 homozygotes and 5 heterozygotes); the missense mutation in the exon 15 (3 homozygotes and 9 heterozygotes); and the Int14A/D442G in 6 compound heterozygotes. ApoE-poor and apoE-rich HDL-cholesterol levels were elevated significantly in all genotypic groups with the decrease in CETP activity, indicating that both types of HDL-cholesterol can be a substrate for CETP. However, an unchanged or only slightly increased serum apoA-II level in each genotype indicated that the HDL particles with apoA-II are relatively resistant to CETP-mediated lipid transfer. Serum apoE-rich HDL level was considerably higher in the Int14A homozygotes than in the compound heterozygotes, in spite of similar apoE-poor HDL-cholesterol levels, which may indicate that apoE-rich HDL is a better substrate for CETP than apoE-poor HDL. Although the apoE-rich and apoE-poor HDL subclasses were similar in the accumulation of cholesteryl ester and depletion of triglyceride, the accumulation of free cholesterol was unique to apoE-rich HDL, indicating inhibited cholesterol esterification on this lipoprotein. Clinical laboratories should be aware of the discrepancy in HDL-cholesterol measurements that comes from the different recoveries of apoE-rich HDL using commercial reagents. In conclusion, CETP deficiency causes considerable quantitative and compositional changes in HDL subclasses, reflecting a significant physiological role for CETP in HDL metabolism.—**Chiba, H., H. Akita, K. Tsuchihashi, S-P. Hui, Y. Takahashi, H. Fuda, H. Suzuki, H. Shibuya, M. Tsuji, and K. Kobayashi,** *Quantitative and compositional changes in high density lipoprotein subclasses in patients with various genotypes of cholesteryl ester transfer protein deficiency. J. Lipid Res.* 1997. **38:** 1204–1216.

**Supplementary key words** apoA-I • apoA-II • polyethylene glycol • polyanion • dextran sulfate • phosphotungstate • hyperalphalipoproteinemia • cholesterol • low density lipoproteins

Cholesteryl ester transfer protein (CETP) is a hydrophobic glycoprotein ($M_r$ 74,000) that catalyzes the net transfer of cholesteryl ester from HDL to triglyceride-rich lipoproteins and the reciprocal net transfer of triglyceride (1, 2). Its gene in chromosome 16q spans 25 kb and contains 16 exons (3, 4). The G to A mutation of the intron 14 splicing donor site (Int14A) of the CETP gene has an allelic frequency of 0.5–0.8% in the general Japanese population and causes a striking elevation in HDL-cholesterol levels (5–9). On the other hand, the Asp 442 to Gly (D442G) in exon 15 has an even higher allelic frequency (3.4–4.6%), and shows a weaker, but significant, effect on HDL-cholesterol levels (8–11). Apart from the above, a rare nonsense mutation, Gln 309 to stop in exon 10, has also been reported (12).

Although previous cases of CETP deficiency have been limited to the Japanese population, we recently found a patient, an ethnic Han Chinese, with the D442G mutation in a small scale study we carried out in China (13). Further, Funke et al. (14) have reported the occurrence of hyperalphalipoproteinemia due to

Abbreviations: apo, apolipoprotein; VLDL, very low density lipoproteins; LDL, low density lipoproteins; HDL, high density lipoproteins; CETP, cholesteryl ester transfer protein; Int14A, G to A substitution at the splicing donor site of intron 14 of the cholesteryl ester transfer protein gene; D442G, aspartic acid to glycine substitution at codon 442 of the cholesteryl ester transfer protein gene; PEG, polyethylene glycol; DNA, deoxyribonucleic acid; EDTA, ethylenediamine tetraacetic acid; LCAT, lecithin: cholesterol acyltransferase.

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two unique CETP gene mutations in Caucasians. Thus, it seems possible that the CETP gene may be an important genetic determinant of serum HDL-cholesterol levels amongst different racial groups. A recent linkage study on Caucasians, in which the strongest linkage to HDL-cholesterol levels was found for the CETP locus amongst genes encoding factors involved in lipoprotein metabolism (15), and other genetic studies have also suggested that it may be a determinant of HDL-cholesterol levels (16–18).

Diagnosis of CETP deficiency is established by confirming *1*) increased serum HDL-cholesterol levels, *2*) decreased serum CETP activity, *3*) CETP gene mutation, *4*) family history, and *5*) absence of other possible causes of HDL-cholesterol elevation, such as chronic alcohol intake (19), insulin-dependent diabetes mellitus (20), nephrosis (21), and drugs (22, 23). Among these, HDL-cholesterol measurement is the most important step in the diagnostic process. For routine measurements of HDL-cholesterol, various precipitation techniques are used to separate HDL from low density lipoproteins (LDL) and very low density lipoproteins (VLDL). We reported significant discrepancies among commercial precipitation reagents found in HDL-cholesterol measurements carried out on a family with Int14A (24). A similar phenomenon in some cholestatic patients, such as those with primary biliary cirrhosis and obstructive jaundice, has also been reported (25–27). With both Int14A mutation and cholestasis, the discrepancies found in the HDL-cholesterol measurements were attributable to the different recoveries of apoE-rich HDL.

We found that a simple precipitation with 13% (w/v) polyethylene glycol (PEG) allowed recovery of total HDL (apoE-rich HDL plus apoE-poor HDL) in the supernatant, while a commercially available polyanionic reagent precipitated apoE-rich HDL and only apoE-poor HDL was recovered in the supernatant (28). PEG precipitates lipoproteins by steric exclusion (29, 30) and, therefore, larger and more hydrophobic lipoproteins precipitate at lower concentrations of PEG. On the other hand, polyanions precipitate lipoproteins through an ionic interaction between polyanions and positively charged lysine and arginine residues of apoB and apoE molecules, in which apolipoprotein composition is critical for precipitation. Thus, our polyanion reagent precipitates VLDL, LDL, and apoE-rich HDL without precipitating apoE-poor HDL, while the 13% PEG solution precipitates VLDL and LDL, leaving smaller and less hydrophobic apoE-rich HDL and apoE-poor HDL in the supernate. Therefore, subtraction of the apoE-poor HDL-cholesterol concentration from total HDL-cholesterol gives the apoE-rich HDL-cholesterol concentration. In a previous study on a family with Int14A (24), we demonstrated a striking increase in serum apoE-rich HDL in the homozygous members using this simple precipitation method.

In the present study, we aim to demonstrate the validity of our precipitation method for CETP-deficient sera, and then to determine the cholesterol concentrations in HDL with and without apoE in subjects with various genotypes of CETP deficiency. We also examine the contribution of CETP activity to the cholesterol concentrations in total HDL, HDL with and without apoE, and LDL. Further, the lipid composition of HDL with and without apoE is studied by applying our precipitation method to other lipid components. These studies should provide useful data for understanding the abnormal HDL metabolism in CETP deficiency and the physiological role of CETP in HDL metabolism.

## MATERIALS AND METHODS

### Subjects

Blood was drawn from patients with hyperalphalipoproteinemia due to CETP deficiency attending our lipid clinic. Their CETP genotypes and ages were as follows: Int14A homozygotes (n = 10, 44 ± 11 (SD) years old) and heterozygotes (n = 5, 49 ± 23 years old); D442G homozygotes (n = 3, 44 ± 5 years old) and heterozygotes (n = 9, 60 ± 19 years old); and Int14A/D442G compound heterozygotes (n = 6; 53 ± 10 years old). Subjects with the Gln 309 to stop mutation were not included in the present study, as this genotype has not yet been found in our laboratory despite the screening of 120 subjects with HDL-cholesterol levels of >100 mg/dl. Healthy volunteers (n = 30; 56 ± 13 years old), whose serum HDL-cholesterol concentrations and CETP activities were normal, were studied as the control group. No subjects were on any medication that would influence lipid metabolism. Blood samples were taken after an overnight fast, and the sera were used for measurements of lipids and CETP activity. Blood samples containing ethylenediamine tetraacetic acid (EDTA)-2Na were used for deoxyribonucleic acid (DNA) analyses. All samples for precipitation experiments and DNA analyses were stored at −20°C until use. No significant effect of freezing and thawing on the measurement of apoE-rich HDL-cholesterol was noted in our previous study (28). Fresh sera were used for electrophoresis and for the study of apolipoprotein distribution by gel filtration. For ultracentrifugation, fresh sera obtained from 7 normal subjects (35 ± 13 years old) were used.

The study protocol was approved by the Internal Review Boards of Hokkaido University School of Medi-

cine. Informed consent was given by each of the participants.

### Lipid and apolipoprotein determinations

Total cholesterol, free cholesterol, triglyceride, and phospholipid in the serum were measured by enzymatic methods using commercial kits (Kyowa Medex Co. Ltd., Tokyo, Japan). Serum cholesteryl ester concentrations were calculated by multiplying the serum esterified cholesterol concentrations, obtained by subtracting free cholesterol from total cholesterol, by 1.72. Apolipoproteins (apoA-I, apoA-II, apoB, apoC-II, apoC-III, and apoE) in serum, and apoE in 13% polyethylene glycol (PEG) supernate were determined by turbidimetric immunoassay (Daiichi Pure Chemicals, Co. Ltd., Tokyo, Japan) in an autoanalyzer (Model Au-510, Olympus, Tokyo, Japan). Apolipoproteins in the column eluate were measured by enzyme immunoassay, as described previously (25).

### Column chromatography for apolipoprotein distribution

Distribution of apolipoproteins among serum lipoproteins was studied by a direct application of fresh serum to a Sepharose CL4B column (1.5 × 92 cm) to avoid a loss of apolipoproteins from lipoprotein particles during ultracentrifugation. One ml of serum each from a normal subject, a patient with partial CETP deficiency (Int14A heterozygote), and a patient with complete CETP deficiency (Int14A homozygote) was applied to the column and eluted with 5 mmol/l Tris-HCl (pH7.4) containing 0.15 mol/l NaCl, 0.27 mmol/l EDTA-2Na, and 3 mmol/l $NaN_3$ at 6°C. Three-ml fractions were collected, and each fraction was analyzed for total cholesterol, apoA-I, apoA-II, apoB, and apoE.

### Determination of total, apoE-rich, and apoE-poor HDL-cholesterol

Serum apoE-poor HDL-cholesterol and apoE-rich HDL-cholesterol concentrations were determined by a combination of two precipitation methods, as described previously (28). Briefly, a sample serum was divided into two parts: one part was mixed with an equal volume of aqueous 13% (w/v) PEG (PEG 6000, Wako Pure Chemicals Industries Ltd., Osaka, Japan), and the other part was mixed with a commercial polyanionic reagent (HDL-C·Daiichi, Daiichi Pure Chemicals Co. Ltd.) composed of 1.8 g/l dextran sulfate, 3 g/l sodium phosphotungstate, and 0.1 mol/l $MgCl_2$. Each mixed solution was allowed to stand for 10 min at room temperature. After centrifugation (2000 *g*, 15 min, room temperature), cholesterol was determined in each supernate. The former procedure (13% PEG) gave apoE-rich HDL-cholesterol plus apoE-poor HDL-cholesterol (designated as total HDL-cholesterol), and the latter gave apoE-poor HDL-cholesterol. ApoE-rich HDL-cholesterol was determined by subtracting the apoE-poor HDL-cholesterol from the total HDL-cholesterol. ApoE in the 13% PEG supernate also analyzed for the study of apoE distribution.

### Validation of the precipitation method for apoE-rich HDL

We previously characterized the interactions between our precipitation reagents and serum lipoproteins from the cholestatic patients with increased apoE-rich HDL concentrations. We here again validated our method for patients with CETP deficiency. The effects of various concentrations of PEG on serum lipoproteins were studied for a patient with complete CETP deficiency (Int14A homozygote). The serum and the same volume of PEG solution (8–15% by weight) were vortex mixed and, after a 15-min standstill, were centrifuged (2000 *g*, 15 min, room temperature). The precipitates were washed once with the same precipitating reagent and solubilized with 1 mol/l NaCl. The supernates and precipitates were electrophoresed in agarose gel film (Corning, Palo Alto, CA), and stained with Fat Red 7B. The recovery of slow α-moving lipoproteins was examined.

Further, we studied the effects of the precipitation on apoE-containing lipoproteins by apoE immunofixation, using serum from a patient with severe CETP deficiency (Int14A/D442G compound heterozygote). For this purpose, the agarose gel, containing electrophoresed lipoproteins (supernate and precipitate), was covered with cellulose acetate membrane immersed in goat anti-apoE (Daiichi Pure Chemicals Co. Ltd.) and allowed to stand for 30 min at room temperature. The gel was then rinsed in 0.15 mol/l NaCl at 4°C overnight. The lipoproteins fixed in the gel were stained with Coomassie brilliant blue.

### Calculation of LDL-cholesterol

The serum LDL-cholesterol concentrations were calculated by the formula of Friedewald, Levy, and Fredrickson (31) (LDL-cholesterol = total cholesterol − HDL-cholesterol − 0.2 × triglyceride), using the total HDL-cholesterol (13% PEG supernate).

### Determination of HDL composition by precipitation

The lipid composition of the total HDL and that of the apoE-poor HDL were determined by measuring the lipids (free cholesterol, cholesteryl ester, triglyceride, and phospholipid) in the 13% PEG supernate and the polyanion supernate, respectively. For apoE-rich HDL, the lipid composition was determined by subtracting the concentrations of each lipid in the polyanion su-

pernate from the concentration in the PEG supernate. Composition was expressed as the fraction of each lipid in the total lipids.

### Ultracentrifugation and column chromatography for lipoprotein composition

Lipoprotein classes were isolated from normal sera by a combination of ultracentrifugation and gel filtration and lipid composition was determined as described previously (25). Briefly, to each serum sample EDTA-2Na, 5′, 5-dithiobis-2-nitrobenzoic acid, and solid KBr (d 1.225 g/ml) were added and the samples were ultracentrifuged in a vertical rotor (Hitachi RPV50T) in a Hitachi 70P-73 ultracentrifuge at 15°C. The floating lipoproteins were collected by pipetting, and concentrated to 2–3 ml by ultrafiltration using Amicon membrane (XM-50). The lipoproteins were then subjected to the CL4B column chromatography as described above. Three-ml fractions were collected under continuous monitoring of the absorbance at 280 nm. According to the elution profile, the fractions of VLDL, LDL, and HDL were pooled and concentrated using Amicon XM-50. Each lipoprotein fraction was subjected to lipid determination.

### Assay of CETP activity

CETP activity was measured by a modification of the method used by Albers, Tollefson, and Chen (32). Briefly, the transfer of $^{14}$C-labeled HDL cholesteryl ester to the d < 1.063 g/ml lipoproteins was measured after incubation of 10 μl of serum for 12 h at 37°C. The HDL and d < 1.063 g/ml lipoproteins were separated by dextran/$MnCl_2$ precipitation, and the radioactivity in the supernate (HDL) was measured. Activity was expressed as a %transfer/10 μ per 12 h. In each experiment, the concentrations of donor and acceptor lipoproteins were adjusted to the condition in which normal serum samples should give a transfer of about 20% of total radioactivity, in order to attain the best linearity of the assay.

### DNA analyses

Genomic DNA was prepared from peripheral buffy coats or total blood using a commercial kit (Blood & Cell Culture DNA kit, Qiagen Inc., Chatsworth, CA). Polymerase chain reactions for the D442G and Int14A mutations were performed as described previously (9). One primer in each primer set was modified to give MspI and NdeI endonuclease cleavage sites, respectively, for the D442G and Int14A mutations. The DNA fragments were analyzed by electrophoresis on 3% NuSieve GTG agarose (FMC Co., Rockland, ME) followed by ethidium bromide staining and visualization using a standard ultraviolet transilluminator.

### Statistical methods

Comparisons of the lipoprotein parameters and CETP activities between the mutants and the controls were evaluated using a Mann-Whitney *U* test. Comparisons of the lipoprotein parameters determined by different methods within the same genotypic group were made using a Wilcoxon signed-rank test. Correlation coefficients were determined by linear regression analysis.

## RESULTS

### CETP activities, serum lipids and apolipoproteins

The CETP activities and the concentrations of lipids and apolipoproteins in serum in the subjects are summarized in **Table 1.** CETP activity was totally lost in the subjects homozygous for Int14A and was very low in the compound heterozygotes. While the Int14A heterozygotes and the D442G homozygotes showed remarkably decreased CETP activities, subjects heterozygous for D442G showed only mild changes. Serum total and free cholesterol, cholesteryl ester, phospholipid, apoA-I, apoC-II, apoC-III and apoE were markedly increased in the Int14A homozygotes and the compound heterozygotes, while little or no change was observed in the other genotypic groups. In spite of the uniform increase in apoA-I in all genotypes, no significant increase was observed in apoA-II, except for the compound heterozygotes. ApoB was significantly decreased only in the Int14A homozygotes, indicating the lack of significant influence of partial CETP deficiency on LDL metabolism.

### Distribution of apolipoproteins among serum lipoproteins

**Figure 1** shows the elution profiles of total cholesterol and apolipoproteins on the CL4B column after a direct application of fresh serum from a normal subject, a patient with partial CETP deficiency (Int14A heterozygote), and one with complete CETP deficiency (Int14A homozygote). The serum lipoprotein parameter levels in these subjects were as follows (in the order of total cholesterol, triglyceride, total HDL-cholesterol, apoA-I, apoA-II, apoB, and apoE): 219, 59, 58, 132, 35, 95, and 3.9 mg/dl for the normal; 236, 119, 74, 157, 36, 103, and 5.9 mg/dl for the Int14A heterozygote; 295, 94, 218, 202, 34, 100, and 16.4 mg/dl for the Int14A homozygote.

For normal serum, the HDL-cholesterol, apoA-I, and apoA-II co-eluted in a roughly monophasic pattern (Fig. 1A, upper), while apoE eluted in both larger HDL and VLDL (Fig. 1A, lower).

TABLE 1. Serum CETP activities and concentrations of serum lipoprotein components in subjects with CETP deficiency

| Traits | Int14A | | D442G | | Int14A/D442G Compound Hetero (n = 6) | Controls (n = 30) |
|---|---|---|---|---|---|---|
| | Homo (n = 10) | Hetero (n = 5) | Homo (n = 3) | Hetero (n = 9) | | |
| | | | % | | | |
| CETP activity | 0 ± 0[b] | 10.9 ± 6.5[b] | 6.4 ± 4.1[b] | 18.3 ± 6.8[b] | 2.6 ± 2.4[b] | 25.6 ± 2.1 |
| | | | *mg/dl* | | | |
| Total cholesterol | 256 ± 35[b] | 217 ± 50 | 204 ± 17 | 209 ± 28[a] | 268 ± 50[b] | 190 ± 26 |
| Free cholesterol | 68 ± 9[b] | 67 ± 21[a] | 53 ± 5[a] | 56 ± 7[b] | 68 ± 13[b] | 45 ± 7 |
| Cholesteryl ester | 327 ± 49[b] | 265 ± 84 | 260 ± 12 | 267 ± 51 | 344 ± 64[b] | 248 ± 34 |
| Triglyceride | 103 ± 27[b] | 106 ± 46 | 49 ± 19 | 56 ± 14 | 55 ± 18 | 71 ± 31 |
| Phospholipid | 298 ± 34[b] | 253 ± 44[a] | 234 ± 28[a] | 232 ± 27[b] | 250 ± 23[b] | 197 ± 24 |
| ApoA-I | 219 ± 39[b] | 190 ± 78[b] | 189 ± 55[a] | 181 ± 35[b] | 250 ± 23[b] | 131 ± 17 |
| ApoA-II | 36 ± 10 | 33 ± 5 | 33 ± 5 | 35 ± 8 | 47 ± 2[b] | 32 ± 5 |
| ApoB | 69 ± 16[a] | 80 ± 18 | 72 ± 18 | 74 ± 15 | 82 ± 18 | 82 ± 14 |
| ApoC-II | 6.2 ± 1.3[b] | 4.7 ± 1.5[b] | 3.2 ± 0.6 | 3.7 ± 1.1[a] | 7.4 ± 1.0[b] | 3.2 ± 1.2 |
| ApoC-III | 21.6 ± 4.8[b] | 12.2 ± 2.2[b] | 8.1 ± 2.8 | 8.3 ± 2.0 | 18.6 ± 2.0[b] | 7.5 ± 2.3 |
| ApoE | 10.8 ± 3.1[b] | 6.6 ± 1.8[a] | 5.0 ± 1.4 | 5.9 ± 1.7[a] | 7.5 ± 3.8[a] | 4.6 ± 1.2 |

Values are mean ± SD.
[a]$P < 0.05$, [b]$P < 0.01$ vs. controls (Mann-Whitney $U$ test).

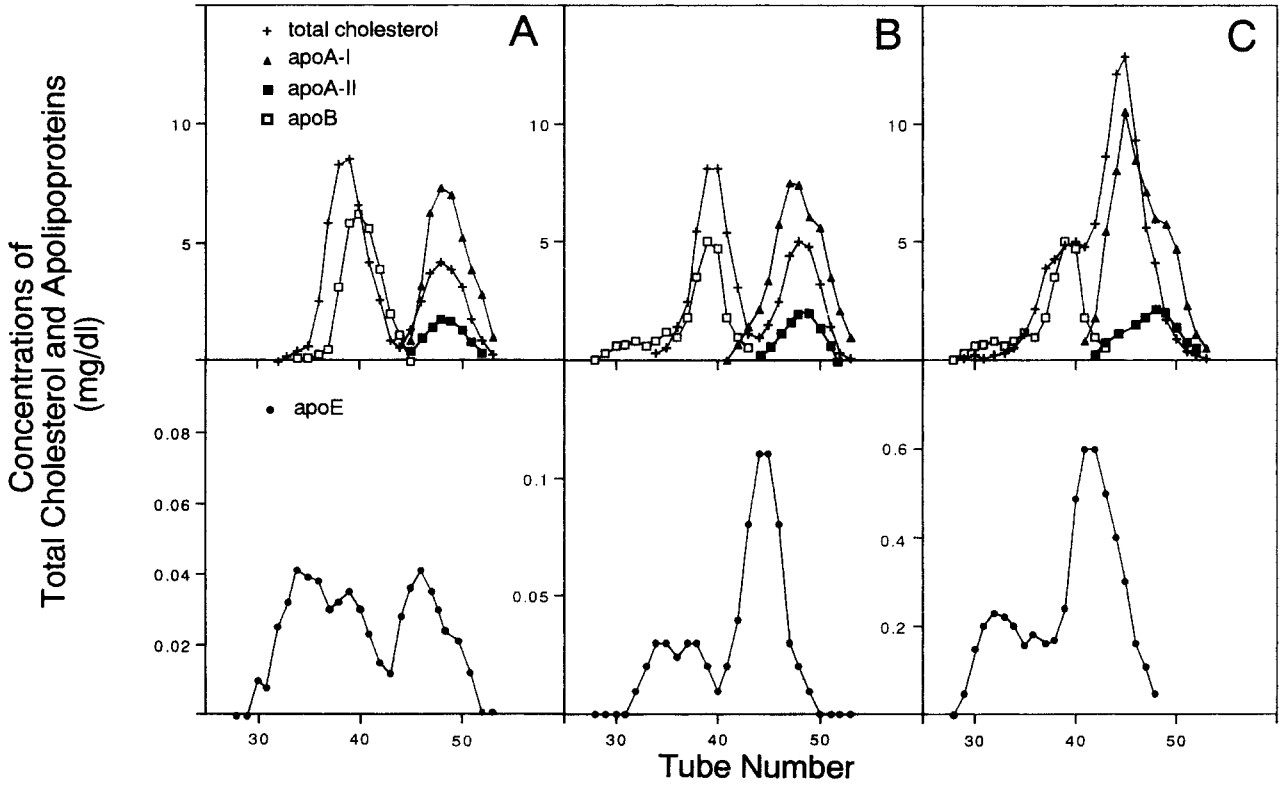


**Fig. 1** Elution profiles of cholesterol and apolipoproteins in native serum from (A) normal subject, (B) a subject with partial CETP deficiency (Int14A heterozygote), and (C) a subject with complete CETP deficiency (Int14A homozygote) on the Sepharose CL4B column. One ml of serum was applied to a Sepharose CL4B column (1.5 × 92 cm) and eluted with 5 mmol/l Tris-HCl (pH 7.4) containing 0.15 mol/l NaCl, 0.27 mmol/l EDTA-2Na, and 3 mmol/l $NaN_3$. Three-ml fractions were collected at 6°C. Cholesterol was determined enzymatically, and apolipoproteins were determined by enzyme immunoassay.

For partial CETP deficiency (Int14A heterozygote), the LDL and HDL peaks were still separated, but a small increase of larger apoA-I-containing lipoproteins was already notable (Fig. 1B, upper). There were no definite changes in ApoA-II distribution. The apoE peak in the larger HDL fraction was enlarged (Fig. 1B, lower).

For complete CETP deficiency (Int14A homozygote), the separation between the LDL-cholesterol and HDL-cholesterol peaks was poor, apparently due to the shift of HDL peak to the front (Fig. 1C, upper). The HDL-cholesterol and apoA-I peaks were strikingly enlarged; in contrast, the LDL-cholesterol and apoB peaks were depressed. ApoA-I eluted in a biphasic pattern. The faster major apoA-I peak overlapped the HDL-cholesterol peak, and the second minor apoA-I peak appeared at the descending limb of the HDL-cholesterol peak, indicating that cholesterol accumulation occurred predominantly in the larger apoA-I-containing particles. ApoA-II elution shifted in the same direction as ApoA-I, but the shift was much less than that for apoA-I, indicating that cholesterol accumulation was small in apoA-II-containing lipoproteins. ApoE-containing HDL particles were more enlarged in particle size (Fig. 1C, lower). A portion of apoE in this fraction no longer overlapped the apoA-I elution, indicating the presence of cholesterol-enriched HDL particles exclusively containing apoE.

### Validity of the method for apoE-rich HDL determination

**Figure 2** shows the effect of various concentrations of PEG on lipoprotein precipitation for the serum from a subject with complete CETP deficiency (Int14A homozygote). As we had found that the apoE-rich-HDL in cholestatic sera was best separated from LDL and VLDL with 13% PEG, we tested the concentrations around 13%. As shown in Fig. 2, the 11–13% PEG solutions successfully recovered the total HDL (α- and slow α-moving lipoproteins) and precipitated the larger lipoproteins. To avoid possible floating of light lipoproteins in hypertriglyceridemic sera, the highest concentration within the range, that is 13%, was chosen for the measurement of total HDL-cholesterol. We have confirmed that this method is safe up to 700 mg/dl of serum triglyceride.

**Figure 3** shows the recovery of apoE-containing lipoproteins in the supernate after the 13% PEG and polyanion precipitations for a patient with severe CETP deficiency (Int14A/D442G compound heterozygote). We did not analyze the polyanion precipitate as we could not solubilize it with any solution. For normal control (Fig. 3a, b), the majority of apoE appeared in the pre-β position, and minor apoE staining was observed in the slow α position. For the compound heterozygote (Fig. 3c, d), there was increased fat staining and dense apoE staining at the slow α-position. The 13% PEG precipitation successfully recovered the α- and slow α-moving lipoproteins in the supernate (Fig. 3e, f), although VLDL and LDL were precipitated under these conditions (Fig. 3g, h). The polyanion precipitation recovered the α-moving lipoproteins in the supernate, but the slow α-moving apoE-containing lipoproteins were not observed there (Fig. 3i, j).

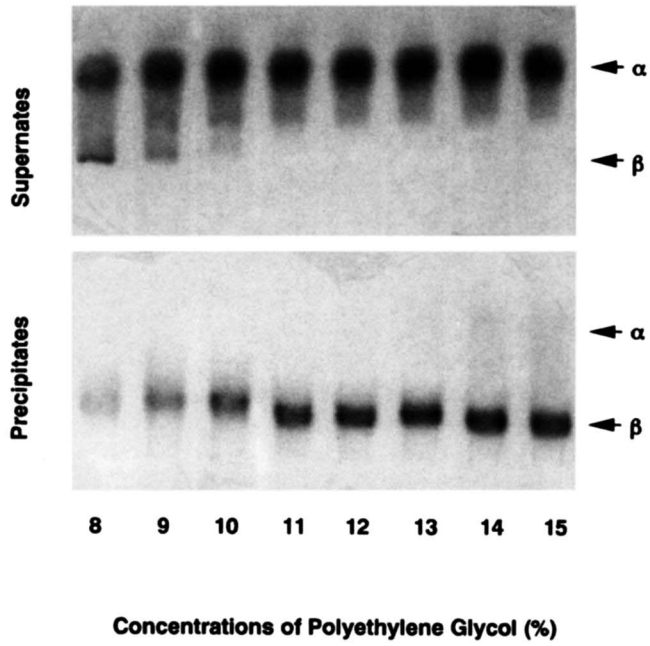


**Fig. 2** Effect of various concentrations of polyethylene glycol on the serum lipoproteins from a subject with complete CETP deficiency (Int14A homozygote). Polyethylene glycol solutions were mixed with the same volume of the serum and centrifuged. Precipitates were solubilized with 1 mol/l NaCl. Fat staining.

The intra-assay and inter-assay coefficients of variation for our measurements of apo-E-rich HDL-cholesterol were 5.3% (n = 20) and 8.2% (n = 12) at 34 mg/dl, respectively.

### HDL-cholesterol and HDL-apoE concentrations by precipitation

Total HDL-cholesterol by the 13% PEG precipitation was strikingly high in the Int14A homozygotes and compound heterozygotes, and moderately high in other genotypic groups (**Table 2**). The absolute cholesterol increase was always larger in apoE-poor HDL than in apoE-rich HDL, but the percentage increase was always larger in the latter in each genotype. A considerable difference in apoE-rich HDL-cholesterol concentration was observed between the Int14A homozygotes and the compound heterozygotes, even though their apoE-poor HDL-cholesterol concentrations were identical.

The absolute apoE concentration in the total HDL

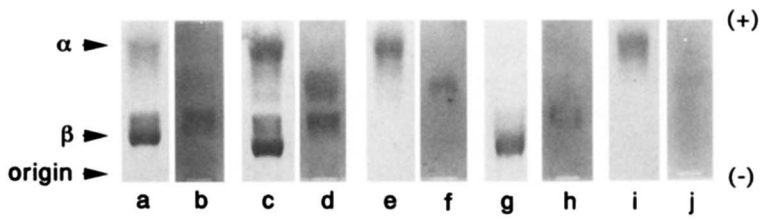


**Fig. 3** Effects of 13% polyethylene glycol and the polyanionic reagent on the serum lipoproteins from a subject with severe CETP deficiency (Int14A/D442G compound heterozygote). Lane's a, c, e, g, i fat staining; lanes b, d, f, h, j: apoE immunofixation; lanes a and b: normal control serum; lanes c and d: the patient's serum; lanes e and f: 13% polyethylene glycol supernate (patient); lanes g and h: 13% polyethylene glycol precipitate (patient); lanes i and j: polyanion supernate (patient).

fraction (13% PEG supernate) was significantly elevated in each genotype, and its increase was roughly parallel to the increase in apoE-rich HDL-cholesterol. On the other hand, the percentage distribution of apoE in the total HDL fraction in each genotype was not significantly different from that for the control, indicating a concomitant increase of apoE content in VLDL. We could not measure apoE in the supernate from the polyanion precipitation as the polyanion reagent caused a significant positive interference in turbidimetric apoE immunoassay, probably due to an interaction with polyanion-binding proteins contained in antiserum and sample serum. The unchanged percentage distributions of apoE in the precipitated lipoproteins, which means the increased absolute apoE content in this fraction, in 13% PEG precipitation might reflect a possible change in polarity of VLDL particles.

### LDL-cholesterol concentrations

LDL-cholesterol concentrations, calculated by the Friedewald's formula using the total HDL-cholesterol (13% PEG precipitation), were 60 ± 39 mg/dl for the Int14A homozygotes (n = 10), 96 ± 43 mg/dl for the Int14A heterozygotes (n = 5), 83 ± 40 mg/dl for the D442G homozygotes (n = 3), 102 ± 26 mg/dl for the D442G heterozygotes (n = 9), 105 ± 46 mg/dl for the Int14A/D442G compound heterozygotes (n = 6), and 116 ± 21 mg/dl for normal control (n = 30). Only the Int14A homozygotes showed a statistically significant decrease ($P < 0.01$).

### Correlation between CETP activities and lipoprotein parameters

CETP activities significantly correlated with the levels of total HDL-cholesterol, apoE-poor HDL-cholesterol, and apoE-rich HDL-cholesterol (**Fig. 4A–C**). The strongest correlation was found for the total HDL-cholesterol. Although the Int14A homozygotes had apparently low LDL-cholesterol levels, the overall correlation between the CETP activities and the LDL-cholesterol levels was not statistically significant (Fig. 4D).

### HDL compositions by precipitation

In both apoE-poor and apoE-rich HDL subclasses, an increase in cholesteryl ester was observed as the most prominent compositional change in each genotypic group (**Table 3**). The decrease in phospholipid and triglyceride in both HDL subclasses was also common among the genotypes. The ratios of neutral lipid (cholesteryl ester + triglyceride) to polar lipid (free

TABLE 2. Serum total, apoE-poor, and apoE-rich HDL-cholesterol concentrations (mg/dl) and serum total HDL-apoE concentrations (mg/dl) in subjects with CETP deficiency

| | | Total HDL (13% PEG Supernate) | | ApoE-Poor HDL (Polyanion Supernate) | ApoE-Rich HDL (Subtraction) |
|---|---|---|---|---|---|
| Genotypes | n | Cholesterol | ApoE | Cholesterol | Cholesterol |
| Int14A homo | 10 | 176 ± 31[b] | 6.4 ± 2.8[b] (60 ± 23%) | 121 ± 24[b] | 55 ± 27[b] |
| Int14A hetero | 5 | 100 ± 36[b] | 2.8 ± 2.2[a] (42 ± 26%) | 84 ± 25[b] | 16 ± 12[b] |
| D442G homo | 3 | 111 ± 35[b] | 2.8 ± 1.4[a] (58 ± 27%) | 93 ± 26[b] | 25 ± 8[b] |
| D442G hetero | 9 | 96 ± 9[b] | 3.1 ± 1.2[b] (54 ± 23%) | 76 ± 8[b] | 21 ± 7[b] |
| Int14A/D442G compound hetero | 6 | 152 ± 10[b] | 5.0 ± 2.5[b] (67 ± 8%) | 121 ± 8[b] | 31 ± 6[b] |
| Controls | 30 | 59 ± 14 | 1.3 ± 0.7 (56 ± 27%) | 52 ± 12 | 7 ± 2 |

Values are mean ± SD. Values in parentheses were obtained by dividing the apoE concentration in the 13% PEG supernate by that in the serum.

[a]$P < 0.05$, [b]$P < 0.01$ vs. controls (Mann-Whitney $U$ test).

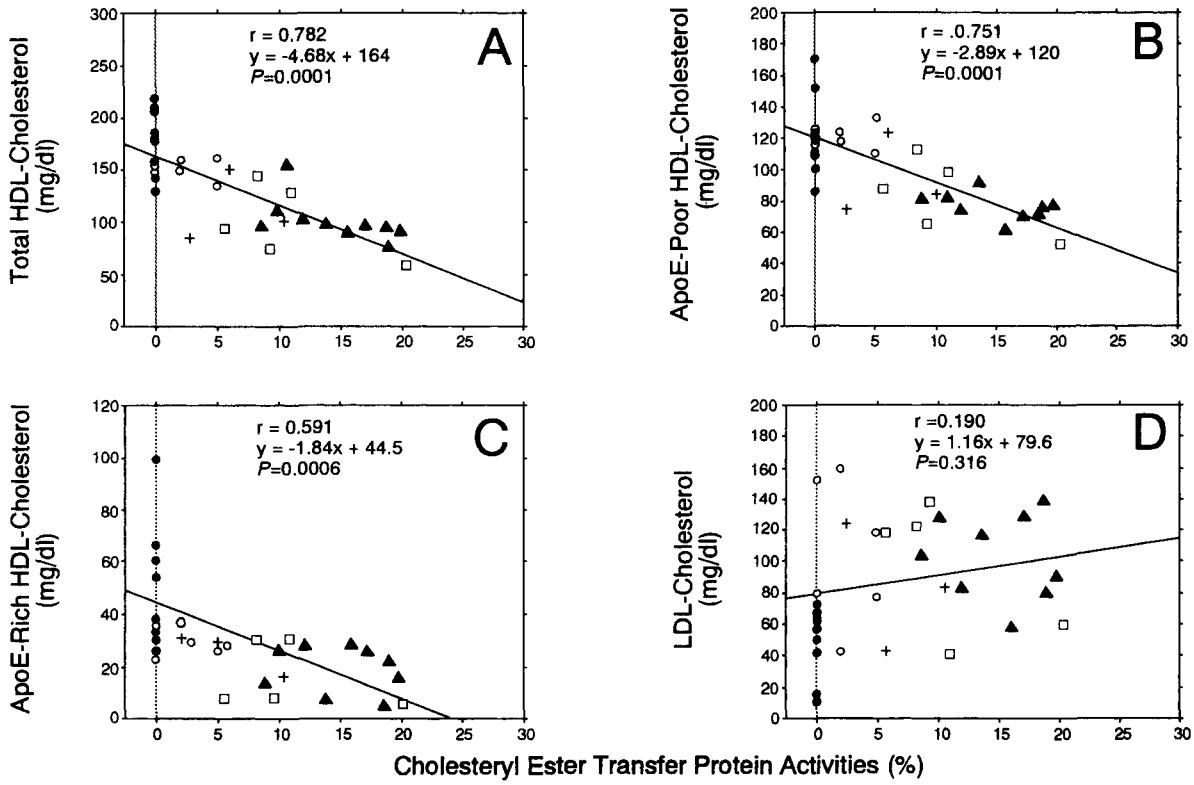


**Fig. 4** Correlations between CETP activity and (A) total HDL-cholesterol level, (B) apoE-poor HDL-cholesterol level, (C) apoE-rich HDL-cholesterol level, and (D) LDL-cholesterol level. (●) Int14A homozygotes; (○) compound heterozygotes (Int14A/D442G); (□) Int14A heterozygotes; (+) D442G homozygote; (▲) D442G heterozygote.

TABLE 3. Lipid compositions (% by weight) of HDL fractions by precipitation in subjects with CETP deficiency

| Genotypes | n | Methods | HDL Fractions | Free Cholesterol | Cholesterol Ester | Triglyceride | Phospholipid | Ratio of Neutral to Polar Lipid |
|---|---|---|---|---|---|---|---|---|
| Int14A homo | 10 | precipitation | total HDL | 6.8 ± 0.8[a] | 47.0 ± 0.9[b] | 0.9 ± 0.5[b] | 45.2 ± 0.9[b] | 0.92 ± 0.05[b] |
| | | | apoE-poor HDL | 5.5 ± 0.9 | 47.1 ± 1.1[b] | 1.1 ± 1.1[b] | 46.3 ± 1.0[b] | 0.93 ± 0.05[b] |
| | | | apoE-rich HDL | 9.8 ± 1.4[b,d] | 46.9 ± 4.1[b,d] | 0.2 ± 0.8[b,c] | 43.0 ± 2.9[b,d] | 0.90 ± 0.13[b] |
| Int14A hetero | 5 | precipitation | total HDL | 5.7 ± 1.3 | 41.2 ± 1.9 | 3.4 ± 1.7 | 49.7 ± 1.4 | 0.80 ± 0.02 |
| | | | apoE-poor HDL | 5.2 ± 0.9 | 41.2 ± 2.6 | 3.2 ± 1.6 | 50.4 ± 2.0 | 0.80 ± 0.04 |
| | | | apoE-rich HDL | 9.3 ± 2.2[b,d] | 42.3 ± 3.2[d] | 4.7 ± 3.8[c] | 46.6 ± 5.2[b,d] | 0.91 ± 0.24 |
| D442G homo | 3 | precipitation | total HDL | 5.7 ± 0.4 | 45.8 ± 5.9 | 2.3 ± 1.0 | 46.2 ± 4.5 | 0.94 ± 0.17 |
| | | | apoE-poor HDL | 4.7 ± 0.3 | 49.0 ± 1.5[a] | 2.2 ± 0.6 | 44.1 ± 0.9[a] | 1.05 ± 0.04[a] |
| | | | apoE-rich HDL | 10.2 ± 1.1[a,c] | 43.1 ± 4.4[c] | 1.6 ± 1.4 | 45.1 ± 3.4[a,c] | 0.98 ± 0.17[a] |
| D442G hetero | 9 | precipitation | total HDL | 6.0 ± 0.9 | 43.6 ± 3.1[a] | 2.8 ± 0.8[a] | 47.6 ± 3.2[b] | 0.86 ± 0.12 |
| | | | apoE-poor HDL | 5.6 ± 1.2 | 44.1 ± 4.0[a] | 2.9 ± 0.9[a] | 47.4 ± 4.3[a] | 0.88 ± 0.16 |
| | | | apoE-rich HDL | 7.7 ± 3.6[a,d] | 46.2 ± 15.3[d] | 1.9 ± 2.1[a,c] | 44.2 ± 13.8[a,d] | 1.02 ± 0.59 |
| Int14A/D442G compound hetero | 6 | precipitation | total HDL | 6.0 ± 0.7 | 48.1 ± 6.0[b] | 1.4 ± 0.4[b] | 44.4 ± 3.7[b] | 0.99 ± 0.18[b] |
| | | | apoE-poor HDL | 5.2 ± 0.9 | 48.5 ± 4.6[b] | 1.3 ± 0.3[b] | 45.1 ± 3.9[b] | 1.00 ± 0.20[a] |
| | | | apoE-rich HDL | 9.7 ± 1.5[b,d] | 47.7 ± 6.8[b,d] | 2.1 ± 1.2[b] | 40.5 ± 6.8[b,d] | 1.04 ± 0.36 |
| Normal | 7 | ultracentrifugation plus column chromatography | HDL | 5.1 ± 1.3 | 38.0 ± 6.9 | 4.4 ± 0.9 | 52.5 ± 5.2 | 0.74 ± 0.11 |
| | | | LDL | 10.2 ± 2.2 | 51.9 ± 3.7 | 11.4 ± 2.1 | 26.5 ± 1.2 | 1.73 ± 0.19 |
| | | | VLDL | 6.3 ± 1.3 | 11.7 ± 3.8 | 63.1 ± 4.0 | 19.0 ± 2.4 | 3.00 ± 0.46 |

Neutral lipid, cholesteryl ester plus triglyceride; polar lipid, free cholesterol plus phospholipid. Values are mean ± SD.
[a] $P < 0.05$, [b] $P < 0.01$ vs. normal HDL (Mann-Whitney $U$ test).
[c] $P < 0.05$, [d] $P < 0.01$ vs. apoE-poor HDL for the same genotype (Wilcoxon signed-rank test).

cholesterol + phospholipid) increased up to the intermediate level between those for the control HDL and LDL fractions, apparently due to the accumulation of cholesteryl ester. The ratios, however, were not significantly different among the HDL subclasses within the same genotype. Instead, increased free cholesterol was found to be a unique characteristic for apoE-rich HDL in all CETP-deficient groups.

## DISCUSSION

ApoE-rich HDL has several characteristics that are not found in apoE-poor HDL, such as high-affinity binding to LDL receptors and remnant receptors (33) and an inhibitory effect on agonist-induced platelet activation (34, 35). Wilson et al. (36) reported that the inverse relationship between HDL-cholesterol and coronary risk might be largely attributable to apoE-rich HDL, and suggested a significance of apoE-rich HDL-cholesterol as a predictor for subjects at coronary risk. Thus, a separate monitoring of apoE-rich and apoE-poor HDL subclasses may provide useful clues as to whether hyperalphalipoproteinemia due to CETP deficiency is anti-atherogenic or atherogenic in nature.

Previous studies have reported an elevation in serum apoE level in patients with CETP deficiency (7–11, 37, 38). Koizumi et al. (38) found that apoE resides mainly in HDL of larger particle sizes in patients homozygous for CETP deficiency. Yamashita et al. (39) isolated slow α-migrating lipoproteins from the d 1.019–1.063 g/ml fraction in patients with complete CETP deficiency. The isolated lipoproteins were similar in density, particle size, enrichment with apoE and cholesteryl ester, and slow α-migrating nature in agarose gel electrophoresis to HDLc, which had been reported in cholesterol-fed CETP-deficient animals (40). In the present study, an increase in serum apoE was confirmed in most genotypic groups (Table 1). This change was associated with the increase in apoA-I, total cholesterol, and phospholipid. ApoCs, known to distribute more in HDL than VLDL in the absence of hypertriglyceridemia (41), were also increased, which is the same change as seen in cholestatic patients with increased apoE-rich HDL (25). Chromatographic studies demonstrated a remarkable enlargement of HDL particles in complete CETP deficiency (Fig. 1C) and also a mild increase in larger HDL particles in partial CETP deficiency (Fig. 1B), in association with preponderant distribution of apoE in the larger HDL particles in the both cases. Agarose gel electrophoresis showed enhanced staining of apoE in slow α-migrating lipoproteins (Fig. 3). The above findings are consistent with an increase in apoE-rich HDL.

As the density and particle size of apoE-rich HDL overlap those of apoE-poor HDL and LDL, the separation of apoE-rich HDL from other lipoproteins has been usually done by ultracentrifugation combined with Pevikon block electrophoresis (40) or with heparin affinity chromatography (42). These methods, however, are qualitative not quantitative, and therefore our knowledge in terms of the quantity of apoE-rich HDL in CETP deficiency is extremely limited (24). In this study, our unique double precipitation-subtraction method for the measurement of apoE-rich HDL was found to be applicable to CETP-deficient sera (Fig. 2 and 3). As the separation between apoE-rich HDL and the larger lipoproteins (VLDL + LDL) was found to be good in spite of their overlapping particle sizes (Fig. 1), we suggest that the separation largely depends on the surface polarity. The smaller neutral to polar lipid ratios for apoE-rich HDL indicate the larger polarity of this lipoprotein in comparison with LDL and VLDL (Table 3).

ApoE-rich and apoE-poor HDL-cholesterol levels were strikingly increased in the Int14A homozygotes and the Int14A/D442G compound heterozygotes, and were slightly increased in other genotypes (Table 2). This fact and the strong correlation with CETP activities for the total HDL-cholesterol seem to support the idea that the CETP-mediated lipid transfer involves all HDL subclasses, not only a specific subclass (Fig. 4). However, it seems also true that the lipid transfer does not occur equally among HDL subclasses. As shown in Table 2, the Int14A homozygotes has a mean apoE-poor HDL-cholesterol concentration (121 mg/dl) equal to that for the compound heterozygotes, but has a higher apoE-rich HDL-cholesterol concentration (55 mg/dl) than the compound heterozygotes (31 mg/dl). This means that when only a small amount of CETP is present in plasma, cholesterol transfers more preferentially from apoE-rich HDL than from apoE-poor HDL, suggesting that apoE-rich HDL is the better substrate for CETP. Accordingly, Gavish, Yitzchak, and Eisenberg (43) reported that in vivo conversion of $HDL_2$ to $HDL_1$ in rats is more sensitive to lipid transfer reaction than is $HDL_3$ to $HDL_2$ conversion. Groener, van Gent, and van Tol (44) also reported a remarkable reduction in particle size of apoE-containing HDL with much smaller change in size of apoA-I-containing HDL, after injection of rats with partially purified CETP.

Thus, the mechanism of the increased apoE-rich HDL in CETP deficiency can be explained, at least partly, by the reduced conversion of apoE-rich HDL to smaller lipoproteins. It should be noted, however, that the increase occurred in terms of apoE as well as cholesterol. The decrease in polarity on HDL, as indicated by the increased ratios of neutral to polar lipid (Table 3), might accelerate apoE to distribute on HDL. A possible

difference in catabolic clearance rate between apoE on HDL and that on the larger lipoproteins, such as chylomicron remnants and VLDL, might be a cause of the accumulated apoE. Although a normal production rate and substantially slower turn-over rate of apoA-I were reported in complete CETP deficiency (45), such information for apoE is not available. Relating to the high apoE concentrations in CETP deficiency, it is interesting that the rat, a CETP-deficient animal, has plasma apoE levels 5- to 13-times higher than those for humans (46). In this animal, plasma LDL level is extremely low, and therefore, cholesterol delivery to peripheral tissues depends on apoE-rich HDL. It seems worthwhile to test the hypothesis that apoE is synthesized more in CETP-deficient humans to compensate for the diminished cholesterol delivery due to the decrease in CETP and apoB and also to the decreased binding of LDL with the LDL receptor (47). Although the binding capacity of the LDL receptors with apoE-rich HDL isolated from a subject with complete CETP deficiency was reported to be small in comparison with normal LDL (39), it seems unlikely that apoE-rich HDL accumulates by saturating the LDL receptors, because apoB was not found to be increased in CETP-deficient subjects (Table 1).

In the present study, we observed normal apoA-II levels in CETP-deficient subjects, the only exception being compound heterozygotes (Table 1). Chromatographic studies showed much smaller changes in size distribution of apoA-II-containing HDL in partial and complete CETP deficiency (Fig. 1B and 1C). These results seem to suggest that the physiological effect of CETP on the metabolism of apoA-II-containing HDL is small in comparison with that for HDL without apoA-II. Accordingly, a lack of correlation between CETP mass and apoA-II was reported for normolipidemic subjects (48). The unchanged apoA-II level might be explained by the low affinity of CETP for HDL with apoA-II (49), and also by the inhibition of CETP-mediated HDL remodeling by apoA-II (50). However, controversial results were reported for apoA-II metabolism in CETP deficiency: unchanged (8) and increased (8, 37) serum apoA-II levels in complete CETP deficiency, and unchanged (8, 11, 37) and increased (7) apoA-II levels in partial CETP deficiency. Decreased catabolism of apoA-II and increased particle size of apoA-II-containing HDL were reported in complete CETP deficiency (51, 52). The effect of CETP on the metabolism of apoA-II-containing HDL may be difficult to define, as apoA-II can distribute among HDL subpopulations with compositional changes in HDL particles.

Both apoE-rich and apoE-poor HDL subclasses showed similar compositional changes: increased cholesteryl ester, decreased triglyceride, and elevated ratio of neutral to polar lipid (Table 3). Although the accumulation of free cholesterol was found to be a unique feature of apoE-rich HDL in CETP deficiency, its mechanism is puzzling, as free cholesterol transfer among lipoproteins is mediated by a rapid CETP-independent mechanism (1). In a previous study by Koizumi et al. (53), increased free cholesterol in HDL was reported for subjects homozygous for Int14A, but no discussion was given on this phenomenon. Chajek, Aron, and Fielding (54) reported that LCAT activity on liposomal surface was directly inhibited by cholesteryl ester. Although we have reported normal LCAT activities in two Int14A homozygotes using a commercial LCAT assay kit (24), in which the substrates were liposome-incorporated lecithin and cholesterol (55), it is still possible that the accumulating cholesteryl ester might inhibit LCAT and increase free cholesterol in apoE-rich HDL. This idea is supported by a previous report that apoE-poor HDL is the better substrate for LCAT than apoE-rich HDL (56).

Increased apoE-rich HDL evokes a problem relevant to clinical chemistry. Several kinds of precipitating reagents for HDL-cholesterol measurement are commercially available, and they are quite different in the recovery of apoE-rich HDL, as described previously (24, 25). Commercial reagents containing PEG only partially recover apoE-rich HDL, because their PEG concentrations are higher than 13%. A similar partial recovery of apoE-rich HDL was found in a commercial reagent for isoelectric precipitation (24). On the other hand, commercial reagents containing polyanion(s), such as dextran sulfate, sodium phosphotungstate, and heparin, precipitate apoE-rich HDL along with the apoB-containing lipoproteins (40, 57). Inazu et al. (8) described a heparin-$Ca^{2+}$ precipitation that results in an estimation of HDL-cholesterol that is 4–11% lower than that by ultracentrifugation in samples with HDL-cholesterol levels of ≥120 mg/dl. Thus, true total HDL-cholesterol concentration, which is best determined by 13% PEG, is not available using any commercial reagents. We do not believe that ultracentrifugation is completely reliable considering the significantly overlapping densities of LDL and HDL in CETP deficiency. This point should be considered when a guideline for the diagnosis of CETP deficiency is discussed.

The significant decrease in serum apoB (Table 1) and LDL-cholesterol in the Int14A homozygotes clearly demonstrated the effect of CETP on LDL metabolism. However, the overall correlation between CETP activities and LDL-cholesterol levels was not significant (Fig. 1D). Previous studies have also reported a lack of correlation between CETP activities or CETP mass concentrations for apoB levels in CETP-deficient subjects (37), and a lack of a correlation between CETP mass concentrations and LDL-cholesterol levels in normolipidemic

subjects (48). We suppose that the effect of CETP on LDL reaches saturation at a very low level of CETP activity in humans. In this context, it seems unlikely that high CETP activities could contribute to the development of atherosclerosis in humans through an increase of plasma LDL-cholesterol. However, the role of high CETP activity in the development of hyperlipidemia remains to be determined, as an association of enhanced cholesteryl ester transfer and several forms of hyperlipidemia have been reported (58–60). Additionally, we found a methodological problem in the measurement of LDL-cholesterol in CETP-deficient subjects that will be discussed elsewhere.

In conclusion, CETP deficiency causes striking quantitative and compositional changes in HDL subclasses, which indicates a significant physiological role for CETP in the metabolism of HDL. Our present results may suggest preferential cholesterol transfer by CETP in the order of apoE-rich HDL, apoE-poor HDL without apoA-II, and HDL with apoA-II, although further investigation is necessary to reach a conclusion. The precise mechanism of the accumulated apoE-rich HDL and its effect on the development of atherosclerosis remain to be determined.

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## REFERENCES

1. Morton, R. E., and D. B. Zilversmit. 1982. Purification and characterization of lipid transfer protein(s) from human lipoprotein-deficient plasma. *J. Lipid Res.* **23:** 1058–1067.
2. Hesler, C. B., T. L. Swenson, and A. R. Tall. 1987. Purification and characterization of a human plasma cholesteryl ester transfer protein. *J. Biol. Chem.* **262:** 2275–2285.
3. Lusis, A. J., S. Zollman, R. S. Sparkes, I. Klisak, T. Mohandas, D. Drayna, and R. M. Lawn. 1987. Assignment of the human gene for cholesteryl ester transfer protein to chromosome 16q12-16q21. *Genomics.* **1:** 232–235.
4. Agellon, L. B., E. M. Quinet, T. G. Gillette, D. T. Drayna, M. L. Brown, and A. R. Tall. 1990. Organization of the human cholesteryl ester transfer protein gene. *Biochemistry.* **29:** 1372–1376.
5. Brown, M. L., A. Inazu, C. B. Hesler, L. B. Agellon, C. Mann, M. E. Whitlock, Y. L. Marcel, R. W. Milne, J. Koizumi, H. Mabuchi, R. Takeda, and A. R. Tall. 1989. Molecular basis of lipid transfer protein deficiency in a family with increased high-density lipoprotein. *Nature.* **342:** 448–451.
6. Inazu, A., J. Koizumi, T. Haraki, K. Yagi, T. Wakasugi, T. Takegoshi, H. Mabuchi, and R. Takeda. 1993. Rapid detection and prevalence of cholesteryl ester transfer protein deficiency caused by an intron 14 splicing defect in hyperalphalipoproteinemia. *Hum. Genet.* **91:** 13–16.
7. Hirano, K., S. Yamashita, T. Funahashi, N. Sakai, M. Menju, M. Ishigami, H. Hiraoka, K. Kameda-Takeyama, K. Tokunaga, T. Hoshino, K. Kumasaka, K. Kawano, and Y. Matsuzawa. 1993. Frequency of intron 14 splicing defect of cholesteryl ester transfer protein gene in the Japanese general population: relation between the mutation and hyperalphalipoproteinemia. *Atherosclerosis.* **100:** 85–90.
8. Inazu, A., X.-C. Jiang, T. Haraki, K. Yagi, N. Kamon, J. Koizumi, H. Mabuchi, R. Takeda, K. Takata, Y. Moriyama, M. Doi, and A. R. Tall. 1994. Genetic cholesteryl ester transfer protein deficiency caused by two prevalent mutations as a major determinant of increased levels of high density lipoprotein cholesterol. *J. Clin. Invest.* **94:** 1872–1882.
9. Akita, H., H. Chiba, K. Tsuchihashi, M. Tsuji, M. Kumagai, K. Matsuno, and K. Kobayashi. 1994. Cholesteryl ester transfer protein gene: two common mutations and their effect on plasma high density lipoprotein cholesterol content. *J. Clin. Endocrinol. Metab.* **79:** 1615–1618.
10. Takahashi, K., X.-C. Jiang, N. Sakai, S. Yamashita, K. Hirano, H. Bujo, H. Yamazaki, J. Kusunoki, T. Miura, P. Kussie, Y. Matsuzawa, Y. Saito, and A. R. Tall. 1993. A missense mutation in the cholesteryl ester transfer protein gene with possible dominant effects on plasma high density lipoprotein. *J. Clin. Invest.* **92:** 2060–2064.
11. Sakai, N., S. Yamashita, K. Hirano, M. Menju, T. Arai, K. Kobayashi, M. Ishigami, Y. Yoshida, T. Hoshino, N. Kameda-Takemura, and Y. Matsuzawa. 1995. Frequency of exon 15 missense mutation (442 D:G) in cholesteryl ester transfer protein gene in hyperalphalipoproteinemic Japanese subjects. *Atherosclerosis.* **114:** 139–145.
12. Gotoda, T., M. Kinoshita, H. Shimano, K. Harada, M. Shimada, J. Ohsuga, T. Teramoto, Y. Yazaki, and N. Yamada. 1993. Cholesteryl ester transfer protein gene deficiency caused by a nonsense mutation detected in the patient's macrophage mRNA. *Biochem. Biophys. Res. Commun.* **170:** 1346–1351.
13. Akita, H., H. Chiba, S.-P. Hui, Y. Takahashi, K. Matsuno, and K. Kobayashi. 1995. Cholesteryl ester transfer protein deficiency: identification in the Chinese. *Am. J. Med. Genet.* **59:** 399–400.
14. Funke, H., H. Wiebusch, L. Fuer, S. Muntoni, H. Schulte, and G. Assman. 1995. Identification of mutations in the cholesteryl ester transfer protein in Europeans with elevated high density lipoprotein cholesterol. *Circulation.* **90:** I–240.
15. Bu, X., C. H. Warden, Y.-R. Xia, C. DeMeester, D. L. Puppione, S. Teruya, B. Lokensgard, S. Daneshmand, J. Brown, R. Gray, J. I. Rotter, and A. J. Lusis. 1994. Linkage analysis of the genetic determinants of high-density lipoprotein concentrations and compositions: evidence for involvement of the apolipoprotein A-II and cholesteryl ester transfer protein loci. *Hum. Genet.* **93:** 639–648.
16. Kondo, I., K. Berg, D. Drayna, and R. Lawn. 1989. DNA polymorphism at the locus for human cholesteryl ester transfer protein (CETP) is associated with high-density lipoprotein cholesterol and apolipoprotein levels. *Clin. Genet.* **35:** 49–56.
17. Kessling, A., S. Ovellette, O. Bouffard, A. Chamberland, C. Béstard, E. Selinger, M. Xhignesse, S. Lusier-Cac, and J. Davignon. 1991. Patterns of association between genetic

variability in apolipoprotein (apo) B, apoAI-CIII-AIV and cholesteryl ester transfer protein (CETP) gene regions and quantitative variation in lipid and lipoprotein traits: influence of gender and exogenous hormones. *Am. J. Hum. Genet.* **50:** 92–106.
18. Heiba, I. M., C. A. DeMeester, Y. R. Xia, A. Diep, V. T. George, C. T. Amos, S. R. Srinivasan, G. A. Berenson, R. C. Elston, and A. J. Lusis. 1993. Genetic contributions to quantitative lipoprotein traits associated with coronary artery disease: analysis of a large pedigree from the Bogalosa Heart Study. *Am. J. Med. Genet.* **47:** 875–883.
19. Savolainen, M. J., M. Hannuksela, S. Seppänen, K. Kervinen, and Y. A. Kesäniemi. 1990. Increased high-density lipoprotein cholesterol concentration in alcoholics is related to low transfer protein activity. *Eur. J. Clin. Invest.* **20:** 593–599.
20. Kahri, J., P.-H. Groop, G. Viberty, T. Elliot, and M.-R. Taskinen. 1993. Regulation of apolipoprotein A-I-containing lipoproteins in IDDM. *Diabetes* **42:** 1281–1288.
21. Sasaki, J., F. Hara, T. Motooka, S. Naito, and K. Arakawa. 1985. Nephrotic syndrome associated with hyper-high-density-lipoproteinemia potentiated by prednisolone therapy. *Nephron.* **41:** 110–113.
22. Homma, Y., H. Ozawa, T. Kobayashi, H. Yamaguchi, H. Sakane, Y. Mikami, Y. Mikami, and H. Nakamura. 1995. Effects of bezafibrate therapy on subfractions of plasma low-density and high-density lipoproteins and on activities of lecithin:cholesterol acyltransferase and cholesteryl ester transfer protein in patients with hyperlipoproteinemia. *Atherosclerosis.* **106:** 191–201.
23. Homma, Y., H. Ozawa, T. Kobayashi, H. Yamaguchi, H. Sakane, H. Nakamura. 1995. Effects of simvastatin on plasma lipoprotein subfractions, cholesterol esterification rate, and cholesteryl ester transfer protein in type II hyperlipoproteinemia. *Atherosclerosis.* **114:** 223–234.
24. Chiba, H., M. Eto, S. Fujisawa, K. Akizawa, S. Intoh, O. Miyata, K. Noda, K. Matsuno, and K. Kobayashi. 1993. Increased plasma apolipoprotein E-rich high-density lipoprotein cholesterol and its effect on serum high-density lipoprotein cholesterol determination in patients with familial hyperalphalipoproteinemia due to cholesteryl ester transfer activity deficiency. *Biochem. Med. Metab. Biol.* **49:** 79–89.
25. Chiba, H., T. Osaka, N. Iwasaki, H. Suzuki, K. Akizawa, S. Fujisawa, S. Intoh, M. Watanabe, J. Yoshida, K. Matsuno, T. Mitamura, and K. Kobayashi. 1991. Spurious elevation of serum high-density lipoprotein cholesterol in patients with cholestatic liver diseases. *Biochem. Med. Metab. Biol.* **46:** 329–343.
26. Umemori, S., H. Chiba, T. Moriyama, S. Takeda, H. Hosokawa, M. Nobuoka, M. Makino, M. Eto, and K. Akiyama. 1993. Elevation of serum apoE-rich HDL concentration in a patient with cholestatic liver disease. *Jpn. J. Clin. Pathol.* **41:** 307–312. (English abstract)
27. Fujisawa, S., H. Chiba, K. Akizawa, S. Intoh, M. Watanabe, K. Matsuno, and K. Kobayashi. 1993. Rapid alteration in serum lipoprotein profile after bile drainage in a patient with acute bile duct obstruction: contribution of cholestasis to cholesteryl ester-dominant apoE-rich HDL accumulation. *Jpn. J. Clin. Pathol.* **41:** 679–684. (English abstract)
28. Chiba, H., K. Akizawa, S. Fujisawa, T. Osaka-Nakamori, N. Iwasaki, H. Suzuki, S. Intoh, K. Matsuno, T. Mitamura, and K. Kobayashi. 1992. A rapid and simple quantificatin of human apolipoprotein E-rich high-density lipoprotein cholesterol in serum. *Biochem. Med. Metab. Biol.* **47:** 31–37.
29. Iverius, P. H., and T. C. Laurent. 1967. Precipitation of some proteins by the addition of dextran or polyethylene glycol. *Biochim. Biophys. Acta.* **133:** 371–373.
30. Viikari, J. 1976. Precipitation of plasma lipoproteins by PEG-6000 and its evaluation with electrophoresis and ultracentrifugation. *Scand J. Clin. Lab. Invest.* **36:** 265–268.
31. Friedewald, W. T., R. I. Levy, and D. S. Fredrickson. 1972. Estimation of the concentration of low-density lipoprotein cholesterol in plasma, without use of the preparative ultracentrifuge. *Clin. Chem.* **18:** 499–502.
32. Albers, J. J., J. H. Tollefson, and C. H. Chen. 1984. Isolation and characterization of human plasma lipid transfer proteins. *Arteriosclerosis.* **4:** 49–58
33. Mahley, R. W., D. Y. Hui, and T. L. Innerarity. 1981. Two independent lipoprotein receptors on hepatic membrane of dogs, swine, and man. J. Clin. Invest. **68:** 1197–1206.
34. Desai, K., K. R. Bruckdorfer, R. A. Hutton, and J. Owen. 1989. Binding of apoE-rich high-density lipoprotein particles by saturable sites on human blood platelets inhibits agonist-induced platelet aggregation. *J. Lipid. Res.* **30:** 831–840.
35. Higashihara, M., M. Kinoshita, T. Teramoto, S. Kume, and K. Kurokawa. 1991. The role of apoE in inhibitory effects of apoE-rich HDL on platelet function. *FEBS Lett.* **282:** 82–86.
36. Wilson, H. M., J. C. Patel, D. Russel, and E. R. Skinner. 1993. Alterations in the concentration of an apolipoprotein E-containing subfraction of plasma high density lipoprotein in coronary heart disease. *Clin. Chim. Acta.* **220:** 175–187.
37. Yamashita, S., D. Y. Hui, J. R. Wetterau, D. L. Sprecher, J. A. K. Harmony, N. Sakai, Y. Matsuzawa, and S. Tarui. 1991. Characterization of plasma lipoproteins in patients heterozygous for human plasma cholesteryl ester transfer protein (CETP) deficiency: plasma CETP regulates high-density lipoprotein concentration and composition. *Metabolism.* **40:** 756–763.
38. Koizumi, J., H. Mabuchi, A. Yoshimura, I. Michishita, M. Takeda, H. Itoh, Y. Sakai, T. Sakai, K. Ueda, and R. Takeda. 1985. Deficiency of serum cholesteryl-ester transfer activity in patients with familial hyperalphalipoproteinemia. *Atherosclerosis.* **58:** 175–186.
39. Yamashita, S., D. L. Sprecher, N. Sakai, Y. Matsuzawa, S. Tarui, and D. Y. Hui. 1990. Accumulation of apolipoprotein E-rich high density lipoproteins in hyperalphalipoproteinemic human subjects with plasma cholesteryl ester transfer protein deficiency. *J. Clin. Invest.* **86:** 688–695.
40. Mahley, R. W., and T. L. Innerarity. 1978. Properties of lipoproteins responsible for high affinity binding to cell surface receptors of fibroblasts and smooth muscle cells. *In* Drugs, Lipid Metabolism, and Atherosclerosis. D. Kritchevsky, R. Paoletti, and W. Holmes, editors. Plenum Publishing Corporation, New York, NY. 99–127.
41. Bury, J., and M. Rosseneu. 1985. Enzyme-linked immunosorbent assay for human apolipoprotein C-III. *J. Clin. Chem. Clin. Biochem.* **23:** 63–68.
42. Weisgraber, K. H., and R. W. Mahley. 1980. Subfractionation of human high density lipoproteins by heparin-Sepharose affinity chromatography. *J. Lipid Res.* **21:** 316–325.
43. Gavish, D., O. Yitzchak, and S. Eisenberg. 1987. In vivo conversion of human $HDL_3$ to $HDL_2$ and apoE-rich $HDL_1$ in the rat: effects of lipid transfer protein. *J. Lipid Res.* **28:** 257–267.

44. Groener, J. E. M., T. van Gent, and A. van Tol. 1989. Effect of lipid transfer protein on plasma lipids, apolipoproteins and metabolism of high-density lipoprotein cholestesteryl ester in the rat. *Biochim. Biophys. Acta.* **1002:** 93–100.
45. Ikewaki, K., D. J. Rader, T. Sakamoto, M. Nishiwaki, N. Wakimoto, J. R. Schaefer, T. Ishikawa, T. Fairwell, L. A. Zech, H. Nakamura, M. Nagano, and H. B. Brewer. 1993. Delayed catabolism of high density lipoprotein apolipoproteins A-I and A-II in human cholesteryl ester transfer protein deficiency. *J. Clin. Invest.* **92:** 1650–1658.
46. Chiba, H., T. Mitamura, and H. Matsumiya. 1990. Sandwich enzyme-linked immunosorbent assay of rat apolipoprotein E. *Biochem. Med. Metab. Biol.* **43:** 53–64.
47. Sakai, N., S. Yamashita, K. Hirano, M. Ishigami, T. Arai, K. Kobayashi, T. Funakoshi, and Y. Matsuzawa. 1995. Decreased affinity of low density lipoprotein (LDL) particles for LDL receptors in patients with cholesteryl ester transfer protein deficiency. *Eur. J. Clin. Invest.* **25:** 332–339.
48. Marcel, Y. L., R. McPherson, M. Hogue, H. Czarnecka, Z. Zawadzki, P. K. Weech, M. E. Whitlock, A. R. Tall, and R. W. Milne. 1990. Distribution and concentration of cholesteryl ester transfer protein in plasma of normolipidemic subjects. *J. Clin. Invest.* **85:** 10–17.
49. Moulin, P., M. C. Cheung, C. Bruce, S. Zhong, T. Cocke, H. Richardson, and A. R. Tall. 1994. Gender effects on the distribution of the cholesteryl ester transfer protein in apolipoprotein A-I-defined lipoprotein subpopulations. *J. Lipid Res.* **35:** 793–802.
50. Rye, K. A., K. H. Garrety, and P. J. Barter. 1992. Changes in the size of reconstituted high density lipoproteins during incubation with cholesteryl ester transfer protein: the role of apolipoproteins. *J. Lipid Res.* **33:** 215–224.
51. Duverger, N., D. Rader, K. Ikewaki, M. Nishiwaki, T. Sakamoto, T. Ishikawa, M. Nagano, H. Nakamura, and H. B. Brewer. 1995. Characterization of high-density lipoprotein particles A-I and A-I:A-II isolated from humans with cholesteryl ester transfer protein deficiency. *Eur. J. Biochem.* **227:** 123–129.
52. Ohta, T., R. Nakamura, K. Takata, Y. Saito, S. Yamashita, S. Horiuchi, and I. Matsuda. 1995. Structural and functional differences of subspecies of apoA-I-containing lipoprotein in patients with plasma cholesteryl ester transfer protein deficiency. *J. Lipid. Res.* **36:** 696–704.
53. Koizumi, J., A. Inazu, K. Yagi, I. Koizumi, Y. Uno, K. Kajinami, S. Miyamoto, P. Moulin, A. R. Tall, H. Mabuchi, and R. Takeda. 1991. Serum lipoprotein lipid concentration and composition in homozygous and heterozygous patients with cholesteryl ester transfer protein deficiency. *Atherosclerosis.* **90:** 189–196.
54. Chajek, T., L. Aron, and C. J. Fielding. 1980. Interaction of lecithin: cholesterol acyltransferase and cholesteryl ester transfer protein in the transport of cholesteryl ester into sphingomyelin liposomes. *Biochemistry.* **19:** 3673–3677.
55. Manabe, M., T. Abe, M. Nozawa, A. Maki, M. Hirata, and H. Itakura. 1987. New substrate for determination of serum lecithin: cholesterol acyltransferase. *J. Lipid Res.* **28:** 1206–1215.
56. Marcel, Y. L., C. Vezina, D. Emond, and G. Suzue. 1980. Heterogeneity of human high density lipoprotein: presence of lipoproteins with and without apoE and their roles as substrates for lecithin:cholesterol acyl transferase. *Proc. Natl. Acad. Sci. USA.* **77:** 2969–2973.
57. Burstein, M., H. R. Scholnick, and R. Morfin. 1970. Rapid method for the isolation of lipoproteins from human serum by precipitation with polyanions. *J. Lipid Res.* **11:** 583–596.
58. Tall, A. R., E. Granot, R. Brocia, I. Tabas, C. Hesler, K. Williams, and M. Denke. 1987. Accelerated transfer of cholesteryl esters in dyslipidemic plasma. *J. Clin. Invest.* **79:** 1217–1225.
59. McPherson, R., C. J. Mann., A. R. Tall, M. Hogue, L. Martin, R. W. Milne, and Y. L. Marcel. 1991. Plasma concentrations of cholesteryl ester transfer protein in hyperlipoproteinemia: relation to cholesteryl ester transfer activity and other lipoprotein variables. *Arterioscler. Thromb.* **11:** 797–804.
60. Inazu, A., J. Koizumi, H. Mabuchi, K. Kajinami, and R. Takeda. 1992. Enhanced cholesteryl ester transfer protein activities and abnormalities of high density lipoproteins in familial hypercholesterolemia. *Horm. Metab. Res.* **24:** 284–288.